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in Castration-Resistant Prostate Cancer

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14. ABSTRACT

Purpose and Scope: The purpose of this work is to better understand the mechanisms of resistance to androgen biosynthesis inhibitors in men with castration resistant prostate cancer, and to investigate clinical methods of overcoming resistance.

Key Accomplishments and Findings to date:

- Primary endpoint of Phase II study of Dose-Increased Abiraterone Acetate in Men with mCRPC (PI: Friedlander) met, showing that increase in dose of abiraterone at the time of clinical resistance does not result in second PSA declines.
- A clinical research paper summarizing these findings as well as the CTC work described below was developed and published in Clinical Genitourinary Cancer.
- CTCs were collected in 41 men with abiraterone-naïve mCRPC at baseline on the aforementioned study. Cells have been enumerated for CTCs, CTC clusters, CTCs expressing stem-like and epithelial markers. A trend towards higher CTC counts and lower CTC clusters was observed in patients who were primarily refractory to abiraterone acetate. CD44 expression on CTCs was not correlated with response to abiraterone.
- Array comparative genomic hybridization (aCGH) of CTC data has been performed in CTCs however the genomic data was of an inconsistent nature due to multiple reasons discussed in the body of this report.
- Phase I study of Abiraterone Acetate plus ARN-509 in men with mCRPC (UCSF PI: Friedlander) fully accrued, initial results showing PSA declines in men with treated with prior abiraterone and prior chemotherapy observed, indicating activity of this combination. Results presented at 2015 ASCO Annual Meeting and 2015 AACR annual meeting. Manuscript in preparation, and an industry-sponsored Phase III study based on these findings in underway.
- Substantial career development: Leader of ASCO 2017 Annual Meeting Prostate Cancer committee, multiple ASCO speaking opportunities, NCCN panel member, accepted offer to become Chief of Division of Hematology and Oncology at Zuckerberg San Francisco General Hospital in 2018, and Associate Director of UCSF Helen Diller Family Comprehensive Cancer Center.

15. SUBJECT TERMS

Prostate cancer, castration-resistant prostate cancer, abiraterone, androgens, circulating tumor cells, treatment resistance

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Table of Contents

	<u>Page</u>
Introduction.....	1
Keywords	1
Overall Project Summary.....	1
Key Research Accomplishments.....	12
Conclusion	12
Publications, Abstracts, and Presentations	14
Inventions, Patents and Licenses.....	15
Reportable Outcomes	15
Other Achievements.....	15
References.....	16
Appendices	attached
CC125511 IRB Study Closeout.....	22-26
Curriculum Vitae	27-42
Dose Increased Abiraterone Manuscript.....	43-52
CTC Isolation Manuscript	53-74

INTRODUCTION

Although androgen biosynthesis inhibitors (ABIs) including ketoconazole and abiraterone improve clinical outcomes and prolong survival in men with castration resistant prostate cancer (CRPC), none are curative, and all patients eventually develop resistance followed by disease progression and death. Resistance is hypothesized to result from either increased systemic or tumor androgen production, mutations in the androgen receptor (AR) signaling pathway leading to ligand-independent AR activity, or through AR-independent pathways. The work carried out under this grant aimed to better understand how this therapeutic resistance develops through genomic analysis (gene copy number and gene methylation status) of tumor biopsies and circulating tumor cells (CTCs) taken from men with CRPC. Further, the work here explored whether clinically targeting proposed mechanisms of resistance can improve outcomes in these patients.

KEYWORDS

Prostate cancer, castration resistant prostate cancer, secondary hormonal therapy, circulating tumor cells, treatment resistance.

OVERALL PROJECT SUMMARY

Statement of Work Aim A: Determine whether resistance to androgen biosynthesis inhibitors (ABIs) is mediated by genomic upregulation of androgen synthesis or by autonomous AR function.

To evaluate whether increased androgen synthesis is implicated in androgen biosynthesis inhibitor resistance work has focused on analyzing biospecimens (circulating tumor cells and metastatic biopsies) derived from NCT01637402, a Phase II study of dose-increased abiraterone acetate, an investigator initiated study that forms the backbone of this this grant. In this study patients took standard-dose abiraterone acetate (1000mg daily), and at the time of PSA or clinical progression patients increased the dose of abiraterone (1000mg twice daily). Clinical results of this clinical trial are discussed in Aim B.

Forty-one patients have been accrued to this study and baseline circulating tumor cells were collected on 38 patients (93%) starting initial abiraterone therapy. Additionally 13 men underwent a matched baseline metastatic tumor biopsy. CTCs were recovered in 20 patients progressing on standard-dose abiraterone. As the elevated dose of abiraterone did not result in any PSA declines in the first 14 evaluable patients who progressed and took the increased abiraterone dose (to be discussed more in Aim B below) CTC analysis has been restricted to the baseline CTCs and CTCs at the time of initial progression on standard-dose abiraterone.

CTC enrichment occurred using the VitaCap assay (Vitatex), which enriches circulating cancer cells able to invade into a fluorescently-labeled collagenous matrix. Cells recovered are then enumerated using FACS. Flow-sorted CTCs then undergo lysis and DNA extraction, followed by batched gene copy number profiling using array comparative genomic hybridization (aCGH). For the metastatic biopsies, tumors are obtained as part of the SU2C-PCF West Coast Dream Team metastatic biopsy acquisition

protocol, and tumor is microdissected from surrounding tissue using laser capture microdissection. DNA and RNA are then isolated for analysis per a formalized protocol. For the purposes of this grant this biopsy data is made available to me for analysis, however the patients undergoing biopsy are consented under a separate IRB-approved protocol and funding for the biopsy analysis is separate from this research training grant.

While initial data derived from CTCs showed clear overlap of aCGH profiles derived from CTCs and matched metastatic biopsy (Figure 1), multiple other aCGH profiles of CTCs did not reveal copy aberrations despite findings of multiple copy aberrations in metastatic tumors. This discordance unfortunately created a significant challenge in terms of the analysis of work originally planned in this Aim. Reasons for this discrepancy and strategies to overcome these are discussed below.

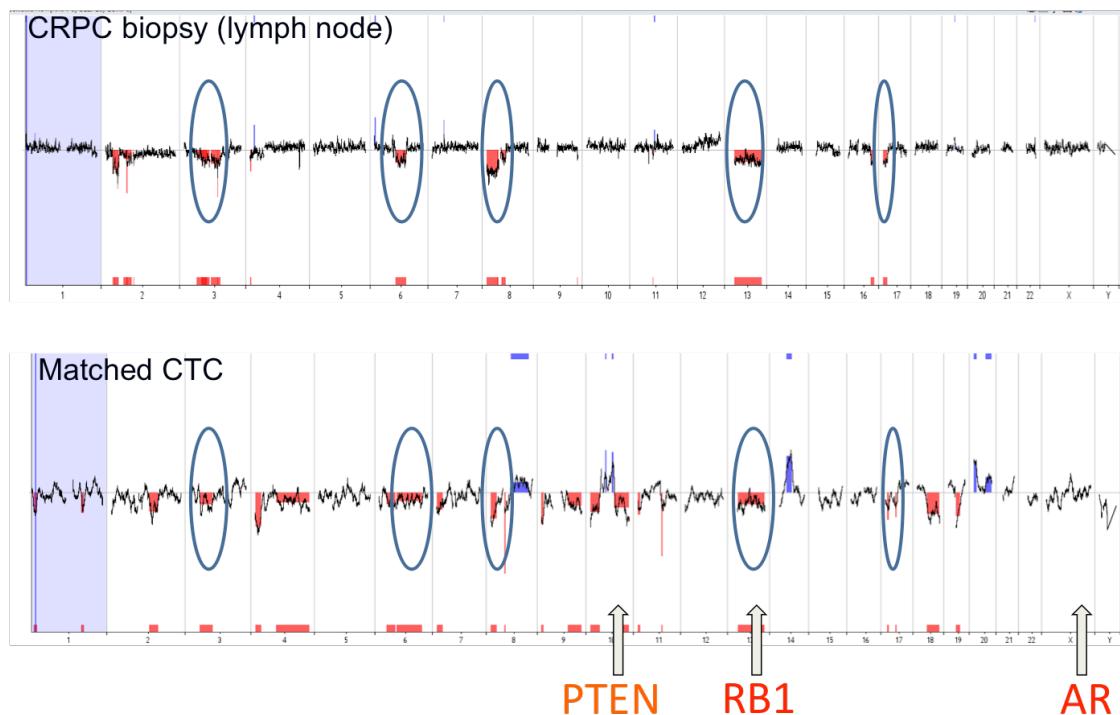


Figure 1: Successful array CGH of CTC and matched metastatic biopsy. Ovals show overlap in multiple segments of chromosomal copy loss. PTEN and RB1 are deleted in both samples. Androgen receptor is copy-normal (wild-type) in both samples.

While the discordance in genomic results between CTCs and metastatic biopsy may reflect heterogeneity in the CTC pool (i.e. metastases from less copy-aberrant primary intact tumors versus from more evolved/copy aberrant metastases) another more likely explanation is an insufficient purity of CTC DNA. This may be due to the fact that the Vitatex assay enriches for CTCs based on the ability of cells to invade into a collagenous matrix, but does not exclude invasive leukocytes, macrophages, etc. In our hands we have been able to show that coupling this assay with FACS as described yields high-purity CTCs (in excess of 90%, Table 1). Despite this high purity there are nonetheless

contaminating leukocytes. As we have shown that DNA isolation works best when performed from 10-cell aliquots (data not shown) it may be that these contaminating “normal” cells have significantly impaired our ability to derive actionable genomic information from CTCs enriched on the Vitatex platform. We have developed and published a manuscript summarizing this technique in prostate, lung, and pancreas cancer entitled “An improved CTC isolation scheme for pairing with downstream genomics – demonstrating clinical utility in metastatic prostate, lung, and pancreas cancer” (attached) for which Dr. Friedlander was a senior author.

Purity % (iCTC/WBC)		
	Pre-FACS	Post-FACS
PC3 spike-in	0.02%	92%
CRPC	0.01%	47%
CRPC	0.35%	96.7%
CRPC	1.43%	93.3%

Table 1: CTCs isolated on the Vitatex platform were defined as nucleated (DAPI+), leukocyte-antigen negative (CD45/14-), and showed evidence of uptake of fluorescently-label collagen adhesion matrix (CAM) fragments (CAM+). Cells after initial FACS were re-run to assess purity. Results indicate that flow sorting significantly enriches for leukocyte-antigen negative cells, suggesting high-purity CTCs.

This absence of clear copy aberrations in most samples has created a significant challenge in our ability to dissect genomic mechanisms of resistance using the CTC pool. To address this problem we took a number of approaches. Firstly a genomic analysis of the metastatic biopsies obtained in 13 patients starting on therapy, using RNASeq to explore the transcript levels of androgen synthesis enzymes at baseline as well as by DNA sequencing. This analysis is occurring as part of the “West Coast Dream Team” (WCDT) study funded by the Prostate Cancer Foundation, AACR, and StandUp to Cancer. This analysis took longer than expected due to personnel changes in the team structure, as well as the need to resequence RNASeq for multiple biopsies (due to issues with reproducibility) and therefore the integrated analysis of androgen synthesis enzyme levels for patients who participated in this study has been discontinued. Similarly, DNA sequencing is being pursued over aCGH (as originally described in the PCRP-PRTA grant) due to the increased ability to detect point mutations, indels, as well as genomic translocations, in addition to individual gene copy number. The change from aCGH to DNASeq has taken more time than expected. Because of these delays and the fact that the data for these patients has already been reported on we have decided to close out the CC125511 study with our IRB as no further work will be pursued on the aCGH of these CTCs. (see attached documentation).

To address the challenge of insufficient genomic data from CTCs from this study we sent some blood samples for a second, enrichment-free CTC platform working in conjunction with Epic Biosciences. In this platform blood from patients enrolled on this study is placed on a glass slide without any prior enrichment, and fluorescent antibodies are used to distinguish CTCs from surrounding leukocytes. CTCs are defined as nucleated (DAPI+), cytokeratin positive (CK+), and CD45-; a fourth channel is available for characterization of individual proteins of interest. We have been able to identify the androgen receptor using a fluorescently labeled antibody directed against the AR protein.

We have observed that the AR is expressed in patients who developed acquired resistance to abiraterone (Figure 2), suggesting that downregulation of AR protein is unlikely to be a major mechanism by which abiraterone resistance develops. As AR splice-variantion, in which alternative splicing of AR mRNA leads to a constitutively-active, androgen-independent AR protein, has been shown to have predictive value in understanding abiraterone and enzalutamide resistance¹ work was performed to stain cells isolated on the Epic platform with an AR N-terminal domain antibody to evaluate for the presence of truncated AR protein suggestive of AR splice variants. As only a handful of samples are under investigation we are limited in our ability to draw meaningful conclusions, unfortunately, from this analysis.

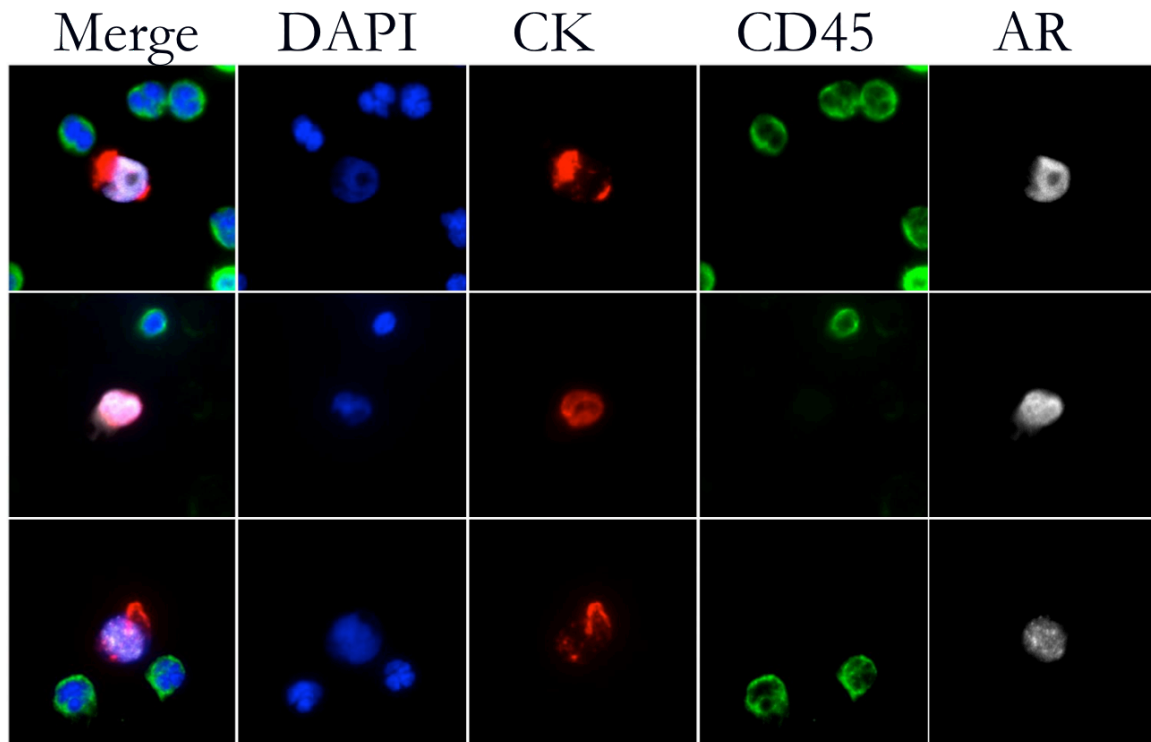


Figure 2: CTCs taken from patients progressing on standard-dose abiraterone acetate isolated using the Epic platform. Each row represents one patient. CTCs are defined as DAPI+/CK+/CD45-. All CTCs continue to express AR protein at the time of abiraterone resistance as evinced by strong cytoplasmic staining.

Similarly, we have initiated a series of studies in which CTCs identified on the Epic platform are individually picked from the slide using an automated cell recovery device, recovered, and genomically profiled. Figure 3 shows data from a series of cell-line experiments showing that PC3 prostate cancer cells are recoverable and informative genomic information is obtainable using aCGH. Given that this technique was started towards the end of the clinical trial there were not enough samples to draw a statistically meaningful conclusion, however this has provided the groundwork for both further study as well as preliminary data for future grant applications to better explore this technique.

In particular, while not funded directly through this grant, a second study exploring the clinical value of detection and genomic analysis of prostate cancer CTCs, taken from men with high risk prostate cancer after radical prostatectomy, has been carried out in conjunction with this grant. Genomic characterization of these CTCs is underway, and a manuscript is in preparation. This study directly stemmed from techniques developed under this Physician Research Training Award.

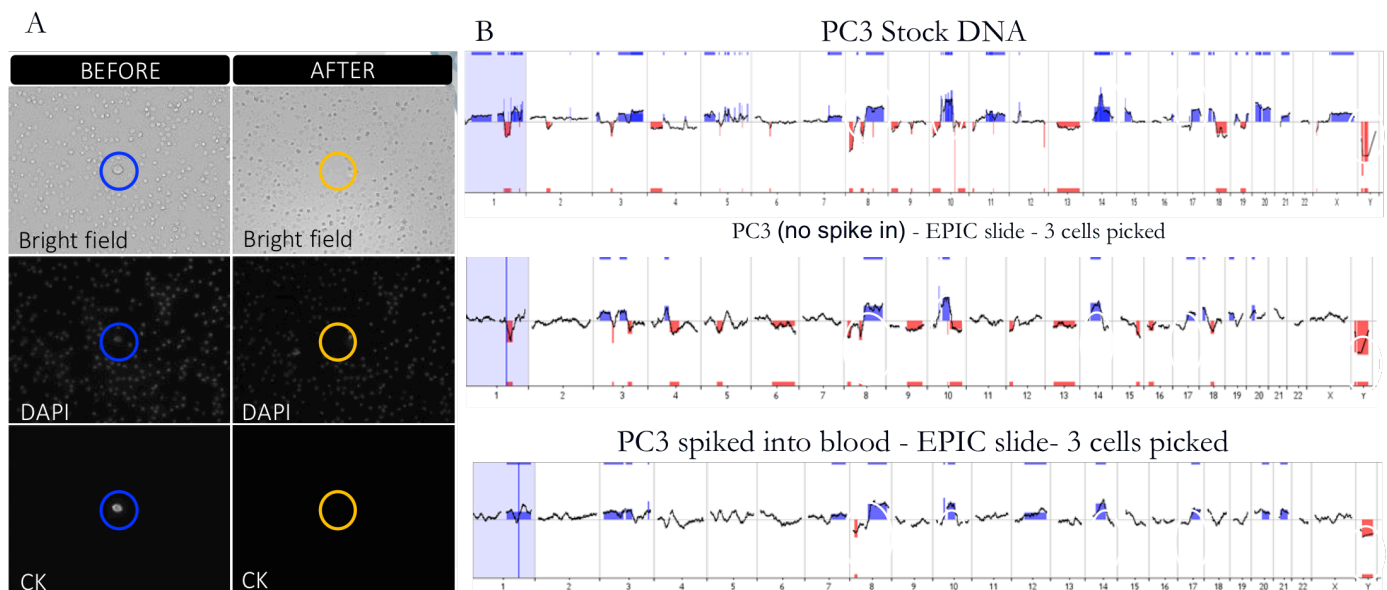


Figure 3. A. Automated cell picking allows for identification, picking, and recovery of single cells. Left column: CK+/DAPI+ CTC prior to cell picking. Right column: CTC removed without disturbing surrounding lymphocytes. B. Genomic analysis of individually recovered CTCs. Top row: aCGH of PC3 stock DNA shows copy gains and losses across the entire genome, with clear deletion of chromosome 8p and amplification of 8q. Middle row: aCGH of PC3 cells (n=3) plated onto Epic slide, then recovered using automated single-cell picking technology. Bottom row: aCGH of PC3 cells (n=3) spiked into healthy donor blood, identified on the Epic platform, then recovered using cell-picking technology. Results show high concordance of copy aberrations across all three experiments.

Given the challenges in assessing the genomics of CTCs from this study a third strategy has been undertaken to evaluate whether baseline CTC subpopulations, as identified immunocytochemically using the original Vitatex platform, can be used as biomarkers to

help understand which patients are likely to have primary resistance or quickly develop acquired resistance to abiraterone. Here we hypothesized that higher CTC counts would predict for either primary resistance or acquired resistance to abiraterone, that the expression of CD44, a putative marker of cancer stem cells, would be associated with more aggressive disease and treatment resistance, and that CTC clusters, defined as 2 or more co-localized CTCs on microscopy, would be a marker of epithelial differentiation (in contrast to solitary CTCs which may represent a more mesenchymal phenotype) and be associated with a higher likelihood of response to AR-targeted therapy.

We observed trends towards both higher CTC counts and lower CTC clusters with lack of PSA response to abiraterone. (Figure 4). It is hypothesized that more CTCs implies a higher disease burden and higher chance for molecular heterogeneity. Similarly it is possible that more CTC clusters implies a more epithelial (as opposed to mesenchymal) phenotype, and that this may lead to more reliance on the AR signaling pathway. While these were not conclusively demonstrated here, they nonetheless are reasonable explanations for the observations and could be investigated further in future studies. Lastly there no clear trend between CD44 positivity and response to abiraterone was observed. This may be due to the fact that the combination CD44 and CD24 or CD133 staining identifies stem-like cells better than CD44 alone², and therefore future work could look at both of these markers in combination.

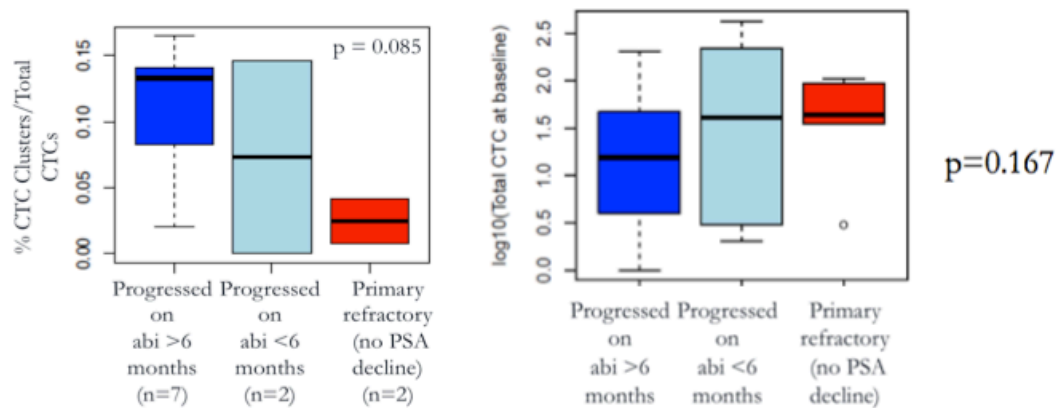


Figure 4: Right: More CTC clusters at baseline associated with longer responses to standard-dose abiraterone. Left: More CTCs associated with higher likelihood of being primarily refractory to treatment with abiraterone.

Components of this data described in Aim A has been presented in poster format at the 2014 ASCO Annual Meeting (Chicago, IL; selected for poster discussion), the 2014 and 2015 ASCO Genitourinary Symposia (San Francisco, CA and Orlando, FL), and at UCSF and the PCF Scientific Retreats. A manuscript describing the CTC results from the dose escalated abiraterone study has been published (Friedlander et al. Clin Genitourin Cancer, 2017, attached), and as noted above a technique paper describing the CTC isolation methods coupled with FACS sorting was also published (Premasekharan et al Cancer Letters 2016, attached)

Statement of Work Aim B: Determine whether resistance to ABIs can be overcome by increased inhibition of androgen synthesis.

To understand whether increased androgen synthesis inhibition can overcome ABI resistance we carried out a Phase II study of Dose-Increased Abiraterone Acetate in Men with mCRPC (PI: Friedlander) in conjunction with investigators at Oregon Health Sciences University and the Knight Cancer Center. The study fully accrued the required 41 patients as described in prior updates. An analysis for the early stopping rule for lack of efficacy was undertaken after a preplanned 14 patients had taken standard dose abiraterone, progressed by PSA or radiographically, and took the elevated dose for at least 12 weeks. While the 1000mg BID dose of abiraterone was well tolerated, and resulted in PSA declines at 12 weeks in 33/41 (80%) of subjects, in this analysis we did not observe any sustained PSA responses to the elevated dose of abiraterone in the first 14 evaluable patients who took this dose. (Figure 6 and attached manuscript) Because of this, and in discussion with the industry sponsor, the elevated-dose arm was closed for lack of efficacy, and it was concluded that escalating the dose of abiraterone at the time of clinical resistance was not clinically warranted. Patients remaining on the standard dose arm have continued to remain on study and are continuing therapy. Currently there are no patients remaining the standard-dose arm.

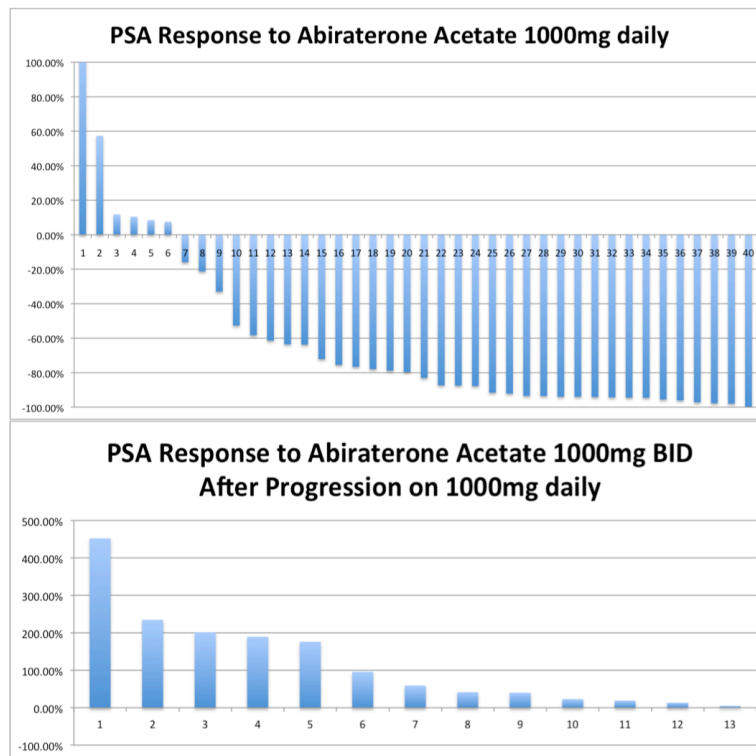


Figure 6: PSA Waterfall plots. Most patients responded to standard dose abiraterone acetate, however no responses were observed after dose-increase in men who progressed on standard dose abiraterone,

Twenty-six patients (63%) taking abiraterone 1000mg daily had baseline and week 4 pharmacokinetic samples evaluable for measurement of abiraterone plasma concentrations. Of these, 7 samples were from patients with primary refractory disease and 19 were from patients who had achieved 30% or greater PSA decline while on standard dose therapy. The plasma abiraterone levels after 4 weeks of standard-dose therapy trended lower among patients with primary refractory disease compared to patients who achieved a consensus-defined PSA decline (median 3.69 ng/mL vs. 7.38 ng/mL, $p=0.073$ Figure 7A). At progression on standard-dose, the plasma levels of abiraterone were also lower among non-responders compared to responders (median 6.00 ng/dl vs. 19.00 ng/dl, $p=0.018$, Figure 7B). Eight out of 13 evaluable patients (61%) taking the increased dose had corresponding increases in the serum level of abiraterone compared to the standard dose, plasma abiraterone concentrations did not rise for the remaining 5 (38%). Overall a non-significant increase in median abiraterone levels was observed in patients taking the increased dose, from 14.2 ng/mL (range 0.80 to 221) at the time of progression on the 1000mg daily dose to 31.5ng/mL (range 5.2 to 148) on the 1000mg BID dose.

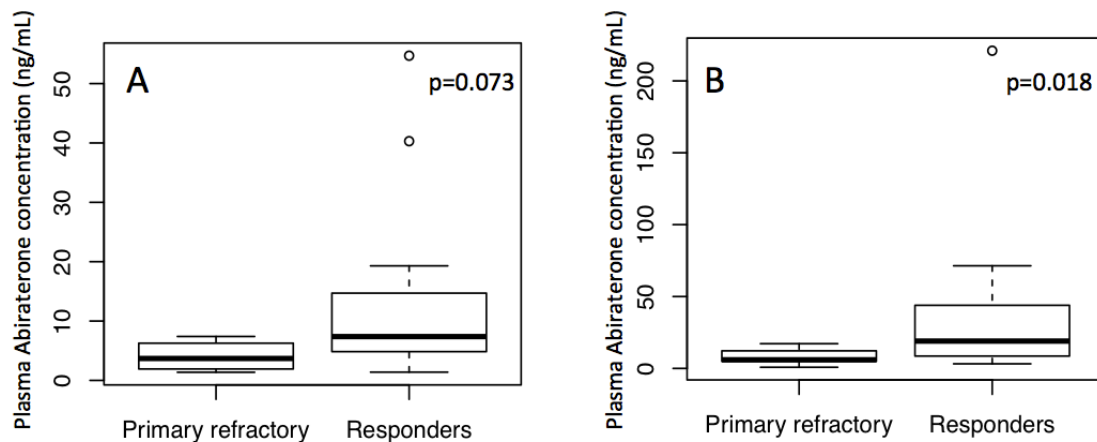


Figure 7: Steady state plasma abiraterone concentrations in primary refractory compared to responders to standard-dose abiraterone therapy after 28 days of therapy (A) and at PSA progression (B).

How to interpret this data? It is known that abiraterone acetate is converted to the active agent abiraterone by CYP3A4 hepatic metabolism and recent work has shown that that conversion of abiraterone to an active metabolite termed D4A is crucial for antitumor activity.³ While D4A levels were not measured in this study we did observe that patients who did not respond after 12 weeks of standard dose therapy had lower plasma abiraterone concentrations after 4 weeks of treatment. Importantly this observation held at the time of PSA progression (Figure 7). This suggests that while acquired resistance may not be pharmacokinetically driven, primary resistance may be, in part, due to inadequate exposure to drug. Unfortunately in this study patients who evinced no PSA

declines after 12 weeks of therapy were removed from study so there is no prospective data to support increasing the dose in these patients, however this approach could be evaluated in a future study. Additionally samples were sent to Dr. Sharifi, the lead author of the D4A report, to see if D4A levels are detectable and have clinical relevance in the residual pK specimens from this study.

In this analysis we observed that levels of abiraterone metabolites (D4A, 3-keto-5 α -Abi, 3 α -OH-5 α -Abi, 3 β -OH-5 α -Abi, 3-keto-5 β -Abi, 3 α -OH-5 β -Abi, and 3 β -OH-5 β -Abi) were measured in 26 patients at baseline and again at time of progression. When compared to patients who had a PSA decline, patients who had primary resistance to AA 1000mg + P had lower baseline 3-keto-5 α -Abi (1.28 vs. 3.58 ng/ml, $p = 0.025$) and 3 β -OH-5 α -Abi (median 0.14 vs. 0.20 ng/ml, $p=0.034$) levels. Interestingly, when comparing patients with primary-resistance, early progression (<6 month response to therapy), and late progression (>6 month response to therapy), baseline 3-keto-5 α -Abi (median 1.28, 5.36 and 3.49 ng/ml respectively, $p=0.042$,) and 3 β -OH-5 α -Abi (median 0.14, 1.02 and 0.19 ng/ml respectively, $p=0.039$) levels remained statistically different between the three groups of patients.

In this study we also observed a trend towards lower baseline plasma DHEA levels in subjects with disease that was primarily refractory to abiraterone compared to patients who subsequently experienced a >30% PSA decline on standard dose therapy (57.50 vs. 80.50 ng/mL, $p=0.43$, Figure 8A). A significant decline in plasma DHEA levels was observed for all patients while on study, however patients who were primarily refractory to standard dose therapy had significantly higher DHEA levels at the time of disease progression compared to patients who acquired resistance to abiraterone after an initial PSA response (13.50 vs. 28.50 ng/mL, $p=0.034$, Figure 8B). Both androstenedione and dihydrotestosterone were suppressed below limit of detection for most patients at the time of disease progression on standard dose therapy, and it was not possible to evaluate whether the increased abiraterone dose further suppressed these levels.

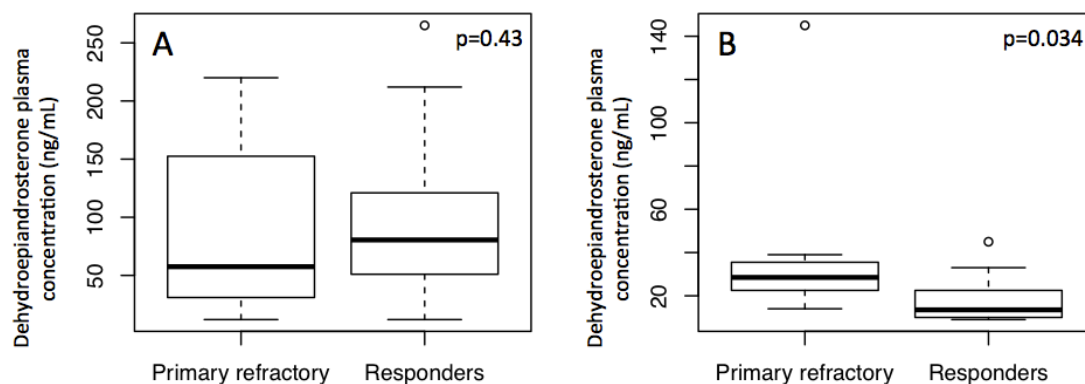


Figure 8: DHEA concentrations at baseline (A) and at the time of resistance to standard dose abiraterone (B)

How to interpret this? High circulating DHEA levels in men with CRPC are thought to indicate an androgen-rich environment, and correlate with a higher likelihood of response to abiraterone.⁴ Here we similarly observed an association between baseline DHEA levels and likelihood of response to abiraterone. While tumor microenvironment DHEA levels were not assayed in this study, the finding that patients refractory to standard dose therapy have lower DHEA levels at baseline may reflect an evolving androgen-independent state in these men. Whether DHEA could be used to risk stratify patients in future studies is worth exploration in a prospectively designed study, and similarly biopsy and interrogation of the AR axis could help better define the biology of tumors that progress in a low DHEA environment.

This data was presented in poster format in part at the 2015 ASCO Genitourinary Symposium (Orlando, FL), as well as intramurally as part of an oral presentation at the 2015 UCSF Prostate Cancer Program Retreat. The published manuscript (Friedlander et al Clin Genitourin Cancer, 2017) is attached.s

Statement of Work Aim C: Determine whether resistance to ABIs can be overcome by AR-targeted therapy.

The clinical trial as described in this grant proposal aims to investigate the value of AR targeted therapy as a way to overcome resistance to androgen biosynthesis inhibitors. A Phase II study of the combination of Abiraterone/prednisone monotherapy, with ARN-509 (a novel AR antagonist) added at the time of resistance in men with abiraterone and chemotherapy naïve metastatic CRPC was developed by Dr Friedlander at UCSF after an agreement was established with Aragon Pharmaceuticals. After the acquisition of Aragon by Janssen the plan for this clinical trial was modified to become a Phase I, open-label, single-arm multicenter study of the combination of abiraterone acetate plus prednisone plus ARN-509 in men with mCRPC (including patients with prior abiraterone and/or prior chemotherapy) with a focus on the pharmacokinetics and drug-drug interaction of these agents. CTC collection from this study has been limited due to the multiple protocol changes as well as the multi-site nature of this study.

This combination study opened at UCSF in late 2014 (UCSF PI: Friedlander). To date twenty-nine patients have been enrolled at all sites, of whom 14 had previous docetaxel, 12 had previous abiraterone, and 12 had previous enzalutamide. Forty-one percent of patients have had a confirmed PSA decline of >50% including in 3 patients who progressed despite prior abiraterone and enzalutamide. Overall the combination has been well tolerated. pK analyses suggest that ARN-509 may lower the pK of abiraterone, while pK of ARN-509 was unaffected compared to historical controls of ARN-509 monotherapy, therefore a second cohort of patients currently being accrued to further explore these findings. The clinical results were presented at the 2015 ASCO Annual Meeting, and have served as the basis for a larger Phase III study currently in planning with industry support. The pK data was presented separately at the 2015 AACR meeting. Currently at UCSF there is one active patient still receiving the combination.

Statement of Work: Training Plan

The major focus of my training has been in the design and conduct of prospective clinical trials and in the analysis of CTC results. To that end I have met weekly with my primary mentor Dr. Charles Ryan and co-mentor Dr. Eric Small, to review progress in the development of the clinical protocols described in the Statement of Work and in the execution and analysis of the clinical data. I have continued meeting twice weekly with my primary scientific advisor Dr. Pamela Paris, who has been integral in helping to think through the laboratory experiments (circulating tumor cell capture and analysis) and to help troubleshoot problems encountered. As noted before both Drs. Phillip Febbo and Carlo Maley left UCSF to pursue academic and industry interests shortly after this grant was awarded. I have been able to maintain contact with Dr. Febbo through email and through discussions and in person at PCF and ASCO meetings. To make up for their departure and the loss of their input regarding the genomic studies I have sought out advice from collaborators in bioinformatics at the University of California, Santa Cruz, whom which our group has developed a close working relationship related to the Stand Up to Cancer metastatic biopsy study described in Aim A. The analysis of tissue biopsies described is underway with their assistance, as well as with Dr. Felix Feng, who was recently recruited to UCSF to assist with the West Coast Dream Team tissue and biomarker analysis.

As the focus of this grant and my career has moved away from the genomic analysis of CTCs to more clinical and translational goals I have deferred some of the coursework in favor of more intensive training with my mentors (both clinical and laboratory). This has resulted in more time to accomplish my goals of becoming an independent clinical investigator. In addition to the studies listed above I have authored multiple clinical trials and grant applications in other GU malignancies including bladder cancer which has been a time intensive but fruitful process. Additionally I have taken on a number of extramural responsibilities including serving as a prostate cancer scientific committee member for the ASCO over the recent years, and served as committee chair in 2017. In this role I led the selection committee, planned and organized all of the academic sessions including selecting abstracts for presentation, speakers and discussants (including selecting the plenary discussant for the LATITUDE study presented at the 2017 Annual Meeting), leading a post-plenary discussion session, and serving as a poster discussant focusing on circulating biomarkers. As attested to in my CV I have also begun serving as a mentor for fellows and students intramurally in addition to serving on other extramural committees including prostate cancer grant review committees for both the DoD and the Prostate Cancer Foundation.

Lastly I have been tapped to lead the Division of Hematology and Oncology at Zuckerberg San Francisco General Hospital, serving as Division Chief starting in 2018, along with becoming an Associate Director of the Helen Diller Family Comprehensive Cancer Center, with a goal of developing a robust research and clinical trials infrastructure at ZSFGH, using lessons learned during the terms of the Physician Research Training Award Grant.

KEY RESEARCH ACCOMPLISHMENTS

- Primary endpoint of Phase II study of Dose-Increased Abiraterone Acetate in Men with mCRPC (PI: Friedlander) met, showing that increase in dose of abiraterone at the time of clinical resistance does not result in second PSA declines. This data was presented at the 2015 ASCO Genitourinary Symposium (Orlando, FL). Manuscript published (see attached)
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- Phase I study of Abiraterone Acetate plus ARN-509 in men with mCRPC (UCSF PI: Friedlander) fully accrued, initial results showing PSA declines in men with treated with prior abiraterone and prior chemotherapy observed, indicating activity of this combination. Results presented at 2015 ASCO Annual Meeting and 2015 AACR annual meeting.
- Integration of both clinical trials with SU2C “West Coast Dream Team” castration-resistant prostate cancer biopsy protocol. Analysis of genomic data (RNAseq, whole exome sequencing) planned, but delayed by resequencing analyses of the WCDT data
- Significant career advancement. Led ASCO 2017 Annual Meeting Prostate Program Committee, served on ASCO 2017 Educational Committee, NCCN panel member (bladder cancer), multiple oral presentations at ASCO Annual Meeting. Promoted to Associate Clinical Professor in July 2017. Tapped to lead the Division of Hematology and Oncology at Zuckerberg San Francisco General Hospital in 2018, and to be Associate Director of the UCSF Helen Diller Family Comprehensive Cancer Center.

CONCLUSION

This Physician Research Training Award has significantly aided me in multiple ways. Our work investigating mechanisms of resistance to AR targeted therapy has yielded important insights into AR biology and the CTC work has show some of the modest value of circulating biomarkers in prostate cancer. I have been able to publish multiple manuscripts related to the work carried out under the grant, and while some of analysis have been challenging, overall we have been able to disseminate both “positive” (clinical trial results) and “negative” (CTC technique work) data. The grant has led to significant

career advancement and has positioned me to lead the Zuckerberg San Francisco General Hospital Division of Hematology and Oncology in 2018 and take on a role as Associate Cancer Center Director at UCSF.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

1. Friedlander TW, Pritchard CC, Beltran H. Personalizing Therapy for Metastatic Prostate Cancer: The Role of Solid and Liquid Tumor Biopsies. *Am Soc Clin Oncol Educ Book*. 2017; 37:358-369. PMID: 28561699
2. Friedlander TW, Graff JN, Zejnullahu K, Anantharaman A, Zhang L, Paz R, Premasekharan G, Russell C, Huang Y, Kim W, Aggarwal RR, Lin AM, Fong L, Alumkal JJ, Beer TM, Sharifi N, Alyamani M, Dittamore R, Small EJ, Paris PL, Ryan CJ. High-Dose Abiraterone Acetate in Men With Castration Resistant Prostate Cancer. *Clin Genitourin Cancer*. 2017 Jun 03. PMID: 28655452
3. Premasekharan G, Gilbert E, Okimoto RA, Hamirani A, Lindquist KJ, Ngo VT, Roy R, Hough J, Edwards M, Paz R, Foye A, Sood R, Copren KA, Gubens M, Small EJ, Bivona TG, Collisson EA, Friedlander TW, Paris PL. An improved CTC isolation scheme for pairing with downstream genomics: Demonstrating clinical utility in metastatic prostate, lung and pancreatic cancer. *Cancer Lett*. 2016 Sep 28; 380(1):144-52. PMID: 27343980
4. Anantharaman A, Friedlander TW. Targeting the androgen receptor in metastatic castrate-resistant prostate cancer: A review. *Urol Oncol*. 2015 Dec 16. pii: S1078-1439(15)00555-4. doi: 10.1016/j.urolonc.2015.11.003.
5. Anantharaman A, Friedlander TW. The double-edged sword of ADT: emerging evidence of cardiovascular, pulmonary, and renal risks. *Oncology (Williston Park)*. 2015 Jan;29(1):67-8. PubMed PMID: 25592211.
6. Anantharaman A, Friedlander TW. A Stepping Stone Toward Personalized Oncology: Genomic Analysis of Circulating Tumor Cells to Guide Management of Metastatic Castration-resistant Prostate Cancer. *Eur Urol*. 2015 Aug 13. pii: S0302-2838(15)00712-5. doi: 10.1016/j.eururo.2015.07.053. [Epub ahead of print] PubMed PMID: 26278804.
7. Terence W. Friedlander, MD, Julie Graff, MD, Li Zhang, PhD, Gayatri Premasekharan, PhD, Archana Dilip, MS, Rosa Paz BA, Rahul Aggarwal, MD, Won Kim, MD, Amy M. Lin, MD, Lawrence Fong, MD, Eric J. Small, MD, Pamela L. Paris, PhD, and Charles J. Ryan, MD. A Phase II Study of Increased-Dose Abiraterone Acetate in Patients with Castration Resistant Prostate Cancer (CRPC): Circulating Tumor Cell Correlatives. Oral abstract presented at 2015 UCSF Prostate Cancer Program Retreat; September 2015; San Francisco, CA.
8. HemOnc Today Men with AR-V7 variant may benefit from taxanes for advanced prostate cancer. July 6th, 2015. Available at <http://www.healio.com/hematology-oncology/prostate-cancer/news/online/%7Bca85417b-9e66-4b4d-ad80->

9. Edwin M. Posadas, Kim N. Chi, Ronald De Wit, Maja J. De Jonge, Gerhardt Attard, Terence W. Friedlander, Margaret K. Yu, Peter Hellemans, Caly Chien, Charlene Connelly Abrams, Martha Gonzalez, Geralyn Carol Trudel, Vijay Chauhan, Juhui James Jiao, Fred Saad. Phase 1b study of ARN-509 with abiraterone acetate (AA) and prednisone (P) in patients (pts) with metastatic castration-resistant prostate cancer (mCRPC). Poster presented at: 2015 ASCO Annual Meeting; June 2015; Chicago, IL. J Clin Oncol 33, 2015 (suppl; abstr 5028)
10. Edwin M. Posadas, Kim N. Chi, Ronald de Wit, Maja JA de Jonge, Gerhardt Attard, Terence Friedlander, Margaret Yu, Peter Hellemans, Caly Chien, Charlene Abrams, Martha Gonzalez, Géralyn C. Trudel, Vijay Chauhan, Fred Saad. Pharmacokinetics (PK) and safety of ARN-509 with abiraterone acetate (AA) and prednisone (P) in patients (pts) with metastatic castration-resistant prostate cancer (mCRPC). [abstract]. In: Proceedings of the 106th Annual Meeting of the American Association for Cancer Research; 2015 Apr 18-22; Philadelphia, PA. Philadelphia (PA): AACR; Cancer Res 2015;75(15 Suppl):Abstract nr CT302. doi:10.1158/1538-7445.AM2015-CT302
11. Terence W. Friedlander, Julie Nicole Graff, Li Zhang, Rosa Paz, Gayatri Premasekharan, Archana Dilip, Andrew Caleb Hsieh, Rahul Raj Aggarwal, Won Kim, Amy M. Lin, Lawrence Fong, Eric Jay Small, Pamela Paris, Charles J. Ryan. Initial results from a phase II study of increased-dose abiraterone acetate in patients with castration resistant prostate cancer (CRPC). Poster presented at: 2015 Genitourinary Cancers Symposium; February 2015; Orlando, FL. J Clin Oncol 33, 2015 (suppl 7; abstr 188)
12. Friedlander TW, Fong L. The end of the beginning: circulating tumor cells as a biomarker in castration-resistant prostate cancer. J Clin Oncol. 2014 Apr 10;32(11):1104-6. doi: 10.1200/JCO.2013.54.7307. Epub 2014 Mar 10. Erratum in: J Clin Oncol. 2014 Jun 10;32(17):1863. PubMed PMID: 24616311.
13. Terence W. Friedlander, MD Julie Graff, MD, Li Zhang, PhD, Rosa Paz, Evelyn Hang, Andrew Hsieh, MD, Rahul Aggarwal, MD, Won Kim, MD, Amy M. Lin, MD, Lawrence Fong, MD, Eric J. Small, MD, Pamela L. Paris, PhD, and Charles J. Ryan, MD. Initial Results from a Phase II Study of Increased-Dose Abiraterone Acetate in Patients with Castration Resistant Prostate Cancer (CRPC). Poster presented at the 21st Annual Prostate Cancer Foundation Scientific Retreat; October 2014; Carlsbad, CA.
14. Terence W. Friedlander, Gayatri Premasekharan, Vy Ngo, Evelyn Hang, Julie Nicole Graff, Charles J. Ryan, Pamela Paris. Molecular and genomic characterization of invasive circulating tumor cells (iCTCs) from men with metastatic castration-resistant prostate cancer (mCRPC). 2014 ASCO Annual Meeting; June 2014; Chicago, IL. Selected for poster discussion. J Clin Oncol 32:5s, 2014 (suppl; abstr 11013)

15. Terence W. Friedlander, Gayatri Premasekharan, Vy Ngo, Shaun Doty, Anna Harris, Michael Winter, Elizabeth Gilbert, Charles Craik, Pamela Paris. Molecular and genomic characterization of invasive circulating tumor cells (iCTCs) from men with metastatic castration-resistant prostate cancer (mCRPC). Poster presented at: 2014 Genitourinary Cancers Symposium; February 2014; San Francisco, CA. J Clin Oncol 32, 2014 (suppl 4; abstr 120)

INVENTIONS, PATENTS, AND LICENCES

None

REPORTABLE OUTCOMES

Two clinical protocols and a laboratory protocol for the work have been developed for this grant. Since the last Update a manuscript for NCT01637402 (Friedlander et al, Clin Genitourin Cancer, 2017, attached) has been published. A second manuscript detailing the CTC techniques used in this work is also published (Premasekharan et al, Cancer Letters, 2016, attached). The initial results of the Phase I study of ARN-509 plus Abiraterone Acetate and prednisone were previously presented at the ASCO 2015 Annual Meeting and at the 2015 AACR annual meeting, both in poster formats. In the last 2 years I have authored multiple editorials about CTCs in prostate cancer in the Journal of Clinical Oncology and European Urology as well as a perspective (about findings related to this scope of work) of the clinical importance of AR-V7 in CTCs published in HemOnc Today. I was lead author on an educational review about circulating biomarkers in prostate cancer. I have co authored a review paper about the importance of androgen signaling in CRPC as well as an editorial about the effects of ADT in men with prostate cancer.

OTHER ACHIEVEMENTS

The CTC development work supported by this grant led to a separate study of circulating tumor cells in bladder cancer, which was published in the Journal of Urology.

See: Alva A, Friedlander T, Clark M, Huebner T, Daignault S, Hussain M, Lee C, Hafez K, Hollenbeck B, Weizer A, Premasekharan G, Tran T, Fu C, Ionescu-Zanetti C, Schwartz M, Fan A, Paris P. Circulating Tumor Cells as Potential Biomarkers in Bladder Cancer. J Urol. 2015 Sep;194(3):790-8. doi: 10.1016/j.juro.2015.02.2951. Epub 2015 Apr 23. PubMed PMID: 25912492.)

Anantharaman A, Friedlander T (co-first author), Lu D, Krupa R, Premasekharan G, Hough J, Edwards M, Paz R, Lindquist K, Graf R, Jendrisak A, Louw J, Dugan L, Baird S, Wang Y, Dittamore R, Paris PL. Programmed death-ligand 1 (PD-L1) characterization of circulating tumor cells (CTCs) in muscle invasive and metastatic bladder cancer patients. BMC Cancer. 2016 Sep 22;16(1):744. PubMed PMID: 27658492; PubMed Central PMCID: PMC5034508.

I similarly received a \$25,000 grant to explore the potential for CTC isolation, genomic profiling, and treatment with personalized therapy for patients with metastatic bladder cancer, through the Cancer League. I am also a coinvestigator on a recently funded R01 grant (R01CA194511-01A1, PI Fong) from the NIH exploring the impact of neoadjuvant immune therapy in bladder cancer.

I have been tapped to lead the Division of Hematology and Oncology at Zuckerberg San Francisco General Hospital with a focus on developing clinical research infrastructure, and will serve as an Associate Director of the Helen Diller Family Comprehensive Cancer Center in 2018.

REFERENCES

1. Antonarakis ES, Lu C, Wang H, Lubner B, Nakazawa M, Roeser JC, Chen Y, Mohammad TA, Chen Y, Fedor HL, Lotan TL, Zheng Q, De Marzo AM, Isaacs JT, Isaacs WB, Nadal R, Paller CJ, Denmeade SR, Carducci MA, Eisenberger MA, Luo J. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. *N Engl J Med*. 2014 Sep 11;371(11):1028-38. doi: 10.1056/NEJMoa1315815. Epub 2014 Sep 3. PubMed PMID: 25184630; PubMed Central PMCID: PMC4201502
2. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res*. 2005 Dec 1;65(23):10946-51. PubMed PMID: 16322242.
3. Li Z, Bishop AC, Alyamani M, Garcia JA, Dreicer R, Bunch D, Liu J, Upadhyay SK, Auchus RJ, Sharifi N. Conversion of abiraterone to D4A drives anti-tumour activity in prostate cancer. *Nature*. 2015 Jul 16;523(7560):347-51. doi: 10.1038/nature14406. Epub 2015 Jun 1. PubMed PMID: 26030522; PubMed Central PMCID: PMC4506215.
4. Kim W, Zhang L, Wilton JH, et al: Sequential use of the androgen synthesis inhibitors ketoconazole and abiraterone acetate in castration-resistant prostate cancer and the predictive value of circulating androgens. *Clin Cancer Res* 20:6269-76, 2014
5. Premasekharan G, Gilbert E, Okimoto RA, Hamirani A, Lindquist KJ, Ngo VT, Roy R, Hough J, Edwards M, Paz R, Foye A, Sood R, Copren KA, Gubens M, Small EJ, Bivona TG, Collisson EA, Friedlander TW, Paris PL. An improved CTC isolation scheme for pairing with downstream genomics: Demonstrating clinical utility in metastatic prostate, lung and pancreatic cancer. *Cancer Lett*. 2016 Sep 28; 380(1):144-52. PMID: 27343980
6. Friedlander TW, Graff JN, Zejnullahu K, Anantharaman A, Zhang L, Paz R, Premasekharan G, Russell C, Huang Y, Kim W, Aggarwal RR, Lin AM, Fong L, Alumkal JJ, Beer TM, Sharifi N, Alyamani M, Dittamore R, Small EJ, Paris PL,

Ryan CJ. High-Dose Abiraterone Acetate in Men With Castration Resistant Prostate Cancer. Clin Genitourin Cancer. 2017 Jun 03. PMID: 28655452

APPENDIX/SUPPORTING DATA

1. UCSF Cancer Center (laboratory) protocol 125511: CC#125511: Determination of Gene Copy Changes Associated with Resistance to Androgen Biosynthesis Inhibitors in Men with Metastatic Castration Resistant Prostate Cancer. IRB approval letter.
2. Curriculum Vitae
3. Manuscript for technique paper: “An improved CTC isolation scheme for pairing with downstream genomics – demonstrating clinical utility in metastatic prostate, lung, and pancreas cancer”
4. Manuscript for dose-increased abiraterone clinical trial: “Increased Dose Abiraterone Acetate in Patients with Castration-Resistant Prostate Cancer: Results of a Phase II Study”.

Study Closeout Report - (Version 1.0)

1.0 Study Closeout Report
1.1 General Information:
<p>Principal Investigator:</p> <p>Terence Friedlander, M.D.</p> <p>Study Title:</p> <p>CC#125511: Determination of Gene Copy Changes associated with Resistance to Androgen Biosynthesis Inhibitors in Men with Metastatic Castration Resistant Prostate Cancer</p> <p>IRB Number:</p> <p>12-08760</p> <p>Expiration Date:</p> <p>06/06/2017</p>
1.2 Lay Summary:
<p>This is a study to determine how resistance to the drug abiraterone acetate occurs in men with prostate cancer. Biopsies and blood samples from men with prostate cancer taking abiraterone will be analyzed to see what genetic changes occur.</p>
1.3 Study Status at Close-out:
<p><input checked="" type="radio"/> Study was completed</p> <p><input type="radio"/> Study was started but closed prior to completion</p> <p><input type="radio"/> Study was not started</p> <p><input type="radio"/> Study is being transferred to another institution</p> <p>Reason study was not started or was closed prior to completion:</p>
1.4 Check the boxes if any of the following apply:
<p><input type="checkbox"/> Local enrollment to the study is ongoing</p> <p><input type="checkbox"/> Local research-related interventions are ongoing</p> <p><input type="checkbox"/> Local participant follow-up is ongoing</p> <p><input type="checkbox"/> Data analysis or manuscript preparation that involves use or access to individually identifiable information is ongoing</p> <p><input type="checkbox"/> Biological specimens associated with individually identifiable information are being maintained in a repository that was approved as part of this study or upon which analysis or research is ongoing (if specimens were transferred to a separate repository that has ongoing CHR approval, then the study may be closed)</p> <p><input type="checkbox"/> Your study has an external sponsor and you have not received permission from your study sponsor to close the study with the CHR</p>
2.0 Study Summary Information
2.1 * Did this study involve the collection, storage, or use of any human biological specimens:

☒ Yes ☐ No

If

YES,

explain what will happen with the specimens at the close of this study:

At the close of the study, all samples will be destroyed.

2.2 Summarize the results of this research project, even if only for the study cohort enrolled locally:

Tissues and circulating tumor cells (CTCs) used in this study were obtained from consenting patients as part of their participation in a clinical trial at UCSF (CC#12551, IRB#12-08740). Patients were treated with abiraterone. Twenty one patients had blood collected for CTC analysis and of the 21 patients, 7 consented to have a biopsy performed. CTC aCGH profiles were analyzed from over 15 patients and investigators have found that some of the profiles closely resemble paired metastases.

2.3 Have there been any presentations or publications resulting from this study since last study renewal?

☐ Yes ☒ No

If **YES**, summarize the content and cite references:

2.4 Recruitment and Enrollment: Was there any participant contact since the date of last renewal?

☐ Yes ☒ No

2.5 Reporting and Summary of Reportable Adverse Events and other Safety Information:

Are you submitting any new or missed AE reporting forms now?

☐ Yes ☒ No

Are you submitting any new or missed DSMB or other multi-center oversight reports now that were not submitted previously?

☐ Yes ☒ No

Were there any other unexpected safety developments that the IRB should know about?

☐ Yes ☒ No

If

YES

to question #3, explain:

If you need to submit an **AE Summary Log**, attach it here:

Version	Title	Category	Last Modified By	Date Last Modified
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No Document(s) have been attached to this form.

2.6 Reporting and Summary of Protocol Violations and/or Protocol Incidents:

Are you submitting any new or missed 10-day Violation/Incident reporting forms now?

☐ Yes ☒ No

Were there any other unexpected developments in study conduct that the IRB should know about (e.g., problems with study activities or participant complaints)?

☐ Yes ☒ No

If

YES

to question #2, explain:

2.7 Study Activity after IRB-Approval Expiration: Please answer the following questions if the IRB study approval has expired.

If the IRB-approval for this study has expired, did any research-related activity(ies) occur during the lapse in approval?

☐ Yes ☐ No

If

YES

, answer the following questions:

Were any participants enrolled during the period of protocol lapse?

☐ Yes ☐ No

Did any other research-related activity(ies) continue during the period of protocol lapse?

☐ Yes ☐ No













How did the approval lapse occur?


What will be done do to prevent this from happening in the future for other studies?

If **YES to either questions #1 or 2**, describe all research activities that continued and whether the activities were done solely for participant safety. Include the number of participants involved and any adverse events or incidents that occurred after expiration of the approval.

Completed Submissions

IRB Study Status:	Closed
Principal Investigator:	Friedlander, Terence, M.D.
Study Number:	12-08760
IRB Study Title:	CC#125511: Determination of Gene Copy Changes associated with Resistance to Androgen Biosynthesis Inhibitors in Men with Metastatic Castration Resistant Prostate Cancer
Expiration Date:	06/06/2017

Reference Number	Request Type	Review Board	View Outcome Letters	Review Process	Meeting Date	Review Outcome	Date Received
042284	Initial Review Submission Packet						
	 Initial Review Submission Packet	IRB Laurel Heights Panel		Returned for Corrections			07/11/2012 09:56:28 AM PDT
	 Submission Correction for Initial Review Submission Packet	IRB Laurel Heights Panel		Forward to Review Board			07/20/2012 10:46:37 AM PDT
	 Submission Correction for Initial Review Submission Packet	IRB Mount Zion Panel		Expedite	07/24/2012	Approved	07/20/2012 11:03:40 AM PDT
068546	Continuing Review Submission Form						
	 Continuing Review Submission Form	IRB Mount Zion Panel		Expedite	06/25/2013	Approved	05/28/2013 04:25:44 PM PDT
088741	Continuing Review Submission Form						
	 Continuing Review Submission Form	IRB Mount Zion Panel		Expedite	06/24/2014	Approved	05/31/2014 10:46:00 AM PDT
138186	Continuing Review Submission Form						
	 Continuing Review Submission Form	IRB Mount Zion Panel		Expedite	07/14/2015	Approved	05/18/2015 10:44:28 AM PDT
166332	Continuing Review Submission Form						
	 Continuing Review Submission Form	IRB Mount Zion Panel		Expedite	06/14/2016	Approved	06/01/2016 10:16:41 AM PDT

193273	Study Closeout Report				
	 Study Closeout Report	IRB Mount Zion Panel	Process Administratively	Approved	05/23/2017 11:34:13 AM PDT

University of California, San Francisco
CURRICULUM VITAE

Name: Terence W. Friedlander, MD

Position: Associate Professor of Clinical Medicine, Step 1
Medicine
School of Medicine

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San Francisco, CA 94143
Voice: 4155146380
Fax: 415-353-7779
Email: terence.friedlander@ucsf.edu

EDUCATION

1995 - 1999	Brown University, Providence RI	BA	Biology
1999 - 2003	New York University Medical School	MD	Medicine
2003 - 2004	University of California, San Francisco	Internal Medicine Internship	Medicine
2004 - 2006	University of California, San Francisco	Internal Medicine Residency	Medicine
2006 - 2007	Utrecht University, Netherlands	MA	Medical Ethics
2007 - 2010	University of California, San Francisco	Fellowship	Hematology/Oncology
2009 - 2010	University of California, San Francisco	Chief Fellow	Hematology/Oncology
2010 - 2011	University of California, San Francisco	Fellowship	Urologic Oncology

LICENSES, CERTIFICATION

2004	Medical Licensure, California (Licence number A88888)
2006	American Board of Internal Medicine, Internal Medicine Certification
2010	American Board of Internal Medicine, Medical Oncology Certification

PRINCIPAL POSITIONS HELD

2011 -	University of California, San Francisco	Associate Clinical Medicine Professor of Medicine
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HONORS AND AWARDS

2000	Herman Goldman Scholarship	NYU Medical School
2003	Spiegel Award for Academic Excellence	NYU Medical School
2003	Alpha Omega Alpha	National Medical Honors Society
2003	Medical Degree with Honors	NYU Medical School
2006	Fulbright Scholarship in Medical Ethics	Netherlands-America Foundation
2010	Young Investigator Award	American Society of Clinical Oncology
2012	Young Investigator Award	Prostate Cancer Foundation
2012	Physician Research Training Award	United States Department of Defense
2012	Travel Award	Advances in Circulating Tumor Cells Conference Foundation
2013	Poster Award	UCSF Annual Prostate Cancer Retreat

KEYWORDS/AREAS OF INTEREST

Prostate Cancer, Bladder Cancer, genomics, microarrays, pharmacogenetics, circulating tumor cells, androgen biosynthesis inhibitors, hormonal therapy, immunotherapy, clinical trials

CLINICAL ACTIVITIES SUMMARY

Attending, Genitourinary Medical Oncology, UCSF: Since 2010 I have seen patients and served as an attending physician in the Mt Zion Genitourinary Medical Oncology clinic twice weekly, seeing patients and supervising rotating fellows, residents and medical students.

Attending, San Francisco General Hospital and SFGH Oncology Clinic: From July 2011 through June 2015 I attended in the SFGH general oncology clinic once weekly, and since July 2011 through the present have attended on the inpatient Oncology Consult service at San Francisco General Hospital 8 weeks out of the year, supervising fellows, residents and medical students.

MEMBERSHIPS

2008 -	American Society of Clinical Oncology
2010 -	American Association of Cancer Researchers

SERVICE TO PROFESSIONAL ORGANIZATIONS

2014 - 2017	American Society of Clinical Oncology (ASCO) Annual Meeting, Prostate Cancer Program Committee	Program Chair 2017. Committee Member 2014-2016.
2016 - 2017	American Society of Clinical Oncology (ASCO) Annual Meeting, Educational Committee	Committee Member
2015 - 2016	Prostate Cancer Foundation (PCF)	Grant reviewer
2015 - 2016	National Comprehensive Cancer Network (NCCN)	Committee Member, Bladder/Penile Cancers Section
2015 - 2016	US Department of Defense, Congressionally Directed Medical Research Program (CDRMP)	Grant reviewer

SERVICE TO PROFESSIONAL PUBLICATIONS

2011 -	Ad hoc referee for the following journals: Journal of Clinical Oncology, Cancer, Clinical Genitourinary Cancer, Urology, European Urology, Urologic Oncology, Prostate Cancer & Prostatic Diseases, Molecular Cancer Therapeutics, Oncotarget, Growth Hormone and IGF Research, Human Mutation, Genes, Journal of Experimental & Clinical Cancer Research, and The Protein Journal	
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INVITED PRESENTATIONS - INTERNATIONAL

2016	10th Annual International Symposium on Minimal Residual Disease, Hamburg, Germany	Poster presentation
2015	European Association of Urology, Annual Meeting, Madrid, Spain	Oral presentation
2015	Global Congress on Prostate Cancer, Rome, Italy	Oral presentation
2013	9th Annual International Symposium on Minimal Residual Disease, Paris, France	Oral Presentation
2012	Advances in Circulating Tumor Cell Conference Committee, Athens, Greece	Oral presentation

INVITED PRESENTATIONS - NATIONAL

2016	Society of Urology Oncology, Annual Meeting	Oral presentation
2016	American Society of Clinical Oncology Annual Meeting	Poster Discussant
2016	American Society of Clinical Oncology Genitourinary Symposium	Poster Presentation
2015	American Society of Clinical Oncology Genitourinary Symposium	Poster Presentation
2015	American Society of Clinical Oncology, Best of ASCO Review Meeting	Oral Presentation

2014	American Society of Clinical Oncology Annual Meeting	Poster Discussion
2014	Prostate Cancer Foundation Annual Scientific Retreat	Poster Presentation
2014	Southwest Oncology Group (SWOG) Spring Meeting	Oral Abstract
2013	Prostate Cancer Foundation Annual Research Symposium	Poster Presentation
2014	American Society of Clinical Oncology Genitourinary Symposium	Poster Presentation
2013	American Society of Clinical Oncology Annual Meeting	Poster Presentation
2012	American Society of Clinical Oncology Genitourinary Symposium	Poster Presentation
2011	American Society of Clinical Oncology Genitourinary Symposium	Oral Plenary Abstract
2010	American Society of Clinical Oncology, Annual Meeting	Poster Presentation
2010	American Society of Clinical Oncology, Genitourinary Symposium	Poster Presentation

INVITED PRESENTATIONS - REGIONAL AND OTHER INVITED PRESENTATIONS

2016	UCSF Prostate Cancer Program Research In Progress Series	Oral Presentation
2016	UCSF Hematology Oncology Research Retreat	Oral Presentation
2015	UCSF Molecular Tumor Board	Oral Presentation : Applied Prostate Cancer Genomics
2015	UCSF Prostate Cancer Program Retreat	Oral Presentation
2015	UCSF Hematology and Oncology Research Retreat	Poster Presentation
2014	SFGH Cancer Awareness Resources and Education	Oral Presentation
2014	UCSF Prostate Cancer Spore Planning Meeting	Oral Presentation
2013	UCSF Prostate Cancer Retreat	Oral Presentation
2013	UCSF Bladder Cancer Support Group	Oral Presentation
2013	UCSF Hematology Oncology Research Retreat	Poster Presentation
2012	UCSF Prostate Cancer Research Retreat	Oral Presentation
2012	UCSF Radiation Oncology Department Grand Rounds	Oral Presentation
2012	SFGH Cancer Awareness Resources and Education	Oral Presentation
2011	UCSF Hematology Oncology Research Retreat	Oral Presentation

2011	UCSF Hematology Oncology Research in Progress Seminar	Oral Presentation
2011	UCSF Bladder Cancer Research Retreat	Oral Presentations
2011	SFGH Cancer Awareness Resources and Education	Oral Presentation
2011	UCSF Prostate Cancer Research Retreat	Poster Presentation
2010	Pfizer Inc. Research Conference	Oral Presentation
2010	UCSF Urologic Oncology Seminar Series	Oral Presentation
2010	UCSF Hematology Oncology Research Retreat	Oral Presentation
2009	SFGH Cancer Awareness Resources and Education	Oral Presentation
2009	Stanford University 11th Annual Multidisciplinary Management of Cancer	Discussant
2009	Cancer and Lymphoma Group B (CALGB) Early Career Investigators Meeting	Oral Presentation

CONTINUING EDUCATION AND PROFESSIONAL DEVELOPMENT ACTIVITIES

2007	UCSF Hematology/Oncology weekly Journal Club
2007	UCSF Hematology/Oncology weekly Clinical Case Conference

GOVERNMENT AND OTHER PROFESSIONAL SERVICE

2015 - 2016	US Department of Defense, Congressionally Directed Medical Research Program (CDRMP)	Grant Reviewer
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SERVICE ACTIVITIES SUMMARY

As an LCE preceptor from I personally precepted and mentor a 3rd year medical student from 2013-2014, 2014-2015, and 2016-2017 for one afternoon per week for ~20 weeks in the GU Medical Oncology clinic at the Cancer Center.

As a Foundations of Patient Care (FPC) Preceptor from 2015-2016 I have precepted and mentored a 1st year medical student for one afternoon per week for ~12 weeks in the GU Medical Oncology clinic at the Cancer Center.

As a lecturer for the UCSF MiniMedical School I have given talks on the management of GU malignancies for a general public audience. These have been video and audio recorded and are available on social media sites including YouTube.

I have served as a small group leader multiple times for the M3 module for second year medical students reviewing case histories as part of the module.

Working with the CARE program at SFGH, giving talks 2-3 times per year I help discuss new trends in oncology management and strategies for survivorship in a Spanish-language community outreach and support program.

As Chief Fellow in 2009-2010 I organized and planned fellowship recruitment and orientation, designed fellows' schedules, implemented year-long performance-improvement projects, served as liaison to program director and division faculty, and mentored junior fellows.

UCSF CAMPUSWIDE

2016 -	Helen Diller Family Comprehensive Cancer Center, Cancer Immunotherapeutics Program	Core Committee Member and Reviewer
2015 -	Helen Diller Family Comprehensive Cancer Center, Clinical Trials Protocol Review Committee (PRC)	Committee Member and Reviewer

SCHOOL OF MEDICINE

2015 - 2016	Foundations of Patient Care (FPC)	Preceptor
2014 -	Urology Oncology Fellowship Program (Dept of Urology)	Interviewer for prospective fellows
2014 -	Hematology and Oncology Fellowship Program	Interviewer for prospective fellows
2014 - 2014	UCSF Mini-Medical School for the Public	Lecturer
2013 - 2017	Longitudinal Clinical Experience (LCE)	Preceptor
2012 -	Hematology and Oncology Fellowship Program	Didactic Lecturer
2009 - 2011	M3 Oncology	Small Group Leader

SCHOOL OF PHARMACY

2015 - 2015	PC152 Drug Discovery and Development	Lecturer
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DEPARTMENTAL SERVICE

2010 - 2011	UCSF Division of Hematology Oncology	Chief Fellow
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COMMUNITY AND PUBLIC SERVICE

2009 - 2016	SFGH Cancer Awareness Resources Education (CARE)	Speaker
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TEACHING SUMMARY

My teaching activities consist of a combination of formal sessions with medical students as a discussion group leader, didactic sessions with the first and second year oncology fellows and medical residents, weekly on-the-go teaching with my LCE medical student during clinic hours, periodic didactics with the SFGH primary care residents and with UCSF Radiation Oncology residents, didactics with research coordinators in the GU Medical Oncology Program, and informal teaching with fellows, residents, and students in the oncology clinics and on the wards.

FORMAL TEACHING

	Academic Yr	Course No. & Title	Teaching Contribution	School	Class Size
	2015 - 2016	Foundations of Patient Care (FPC)	Weekly clinical mentoring with a 1st year UCSF Medical Student	Medicine	1
	2015 - 2015	PC 152 Drug Discovery and Development	Lecturer	Pharmacy	20
	2013 - 2016	Longitudinal Clinical Experience Preceptorship	Weekly clinical mentoring with a 3rd year UCSF Medical Student	Medicine	1
	2013 - 2016	Hematology Oncology Fellowship Didactic Lectures	Introduction to Testicular Cancer	Medicine	6
	2013 - 2016	UCSF GU Medical Oncology Program Research Training	Testis, Kidney, Bladder Cancer didactics.	Medicine	8
	2013 - 2016	SFGH Family Practice Residency Didactic Lectures	Prostate Cancer Review	Medicine	6
	2011 - 2016	Hematology Oncology Fellowship Didactic Lectures	Introduction to Bladder cancer	Medicine	8
	2009 - 2014	M3: Mechanisms, Molecules, and Malignancies	Discussion Group Leader; 2 two hour sessions	Medicine	12
	2011 - 2013	Internal Medicine Residency Noon Lectures	Updates in Prostate Cancer	Medicine	

INFORMAL TEACHING

- 2010 - 2016 Genitourinary Clinic Attending (weekly with fellow, resident, or medical student)
- 2011 - 2016 SFGH Oncology Consult Service (8 weeks, with fellows, residents, and/or medical students)

MENTORING SUMMARY

I served as the principal mentor for Dr. Anantharaman, a third year hematology-oncology fellow, working with the GU Medical Oncology group. Together we investigated the role of circulating tumor cells as biomarkers in prostate and bladder cancer, and published the results of a clinical trials of high dose abiraterone. I have mentored her grant application for an AACR

fellowship, and we published our original research, 2 editorials, and coauthored a review paper on advanced prostate cancer.

Working as an LCE preceptor and mentor from 2013-2016 I meet weekly with a 3rd year UCSF Medical Student, in the GU Medical Oncology clinic to see patients, discuss clinical findings, and help them to develop critical clinical problem solving skills. We also discuss career choices and I provide mentoring in this regard. I similarly have mentored a first year medical student in the Foundations of Patient Care (FPC) in 2015-2016.

Dr. Curro Zambrana was a visiting MD in early 2014, and we worked together weekly in clinic and on a research projects reviewing the UCSF experience with autologous stem cell transplant for advanced germ cell tumors.

Working at Mission Bay I helped supervise and mentor Gayatri Premasekharan, PhD, a post-doctoral fellow in the Paris lab, focusing on the clinical and translation role for circulating tumor cells in prostate cancer.

PREDOCTORAL STUDENTS SUPERVISED OR MENTORED

Dates	Name	Program or School	Mentor Type	Role	Current Position
2016 - 2017	Robert Orynich	UCSF Medical School	Career Mentor, Co-Mentor/Clinical Mentor	LCE Preceptor and Mentor	3rd year UCSF medical student
2015 - 2016	Tiana Woolridge	UCSF Medical School	Career Mentor, Co-Mentor/Clinical Mentor	FPC Preceptor and Mentor	1st year UCSF medical student
2014 - 2015	Jonathan Ostrem	UCSF Medical School	Co-Mentor/Clinical Mentor	LCE Preceptor and Mentor	Internal Medicine Resident at BI Deaconess
2013 - 2014	Max Jan	UCSF Medical School	Career Mentor, Co-Mentor/Clinical Mentor	LCE preceptor and Mentor	Pathology resident at Massachusetts General Hospital

POSTDOCTORAL FELLOWS AND RESIDENTS MENTORED

Dates	Name	Fellow	Mentor Role	Faculty Role	Current Position
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Dates	Name	Fellow	Mentor Role	Faculty Role	Current Position
2015 - 2017	Archana Anantharaman		Research/Scholarly Mentor, Career Mentor, Co-Mentor/Clinical Mentor	Mentor in GU Medical Oncology	Hematology Oncology Fellow
2013 - 2014	Gayatri Premasekharan		Research/Scholarly Mentor, Project Mentor		Post-doctoral fellow

FACULTY MENTORING

Dates	Name	Position while Mentored	Mentor Type	Mentoring Role	Current Position
			Co-Mentor/Clinical Mentor		

VISITING FACULTY MENTORED

2014 - 2014 Curro Zambrana, MD Hospital Infanta Sofia, Madrid, Spain

RESEARCH AND CREATIVE ACTIVITIES SUMMARY

My research is focused on understanding the biology of advanced prostate and bladder cancers and developing novel therapeutics to treat these diseases. Specifically I am interested in understanding the genomics of advanced prostate cancer through the acquisition of castration-resistant biopsies and circulating tumor cells, then using genomic techniques to identify pathways and mechanisms of treatment resistance. Similarly I am interested in developing novel chemotherapeutic and immunotherapeutic strategies to treat both localized and advanced bladder cancer. I also collaborate with basic scientists at UCSF to use circulating tumor cells both as biomarkers of response and resistance to therapy in prostate and bladder cancer, and also as a tool to investigate the basic biology of these tumors.

RESEARCH AWARDS - CURRENT

1. R01CA194511-01A1	Sub-Investigator	5% % effort	Fong (PI)
NIH		2015-07-02	2020-06-30
Immunotherapy of human bladder cancer		\$ 362,569 direct/yr 1	

Immunotherapy has emerged as an important treatment modality for cancer. In contrast to most other cancer treatments, immunotherapy can induce durable clinical response in metastatic disease. Antibodies targeting PD-1 and anti-PD-L1 have shown significant clinical activity in multiple diseases. Importantly, dramatic clinical responses have been seen in patients with the metastatic bladder, a disease in which no new drugs have been approved for over 20 years. The mechanism by which these immunotherapies work in cancer patients is unknown, nor are there robust biomarkers that predict of response to these treatments. We will perform a clinical study administering anti-PD-L1 antibody in patients with localized bladder cancer prior to these patients undergoing planned surgery for their cancer. We propose to study the effects of anti-PD-L1 antibody treatment not just in the blood but within the actual tumor. We will determine whether this immunotherapy increases the number of T cells into the tumor by examining tissues from biopsies obtained before treatment and from the bladder tumor resected following treatment. We will use next generation sequencing to track individual T cell clones in the blood and tumors to also determine whether T cells present in the tumor are being recruited to the tumor site or were already at the tumor. We will study how this treatment activates a systemic immune response. Understanding how these treatments work within patients, including at the level of the tumor tissue, will provide avenues to improve the efficacy of this approach and/or develop biomarkers to identify those patients that can benefit from treatment. Moreover, if significant tumor regression is seen, this treatment approach could transform our approach to localized bladder cancer. Finally, the clinical and laboratory findings derived from this trial may also help transform in how metastatic bladder cancer could be treated and contribute significantly to the design of future clinical trials with P-L1 and/or PD-1 targeted drugs.

Clinical lead, coordination of study procedures, patient accrual, acquisition of tissues, and biobanking.

RESEARCH AWARDS - SUBMITTED

1. In review	PI	20% % effort	Friedlander (PI)
NIH R01 PA-16-160			
Molecular and genetic determinants of sensitivity to PD-L1 inhibition in bladder cancer.			\$ 1M total
This is a pending grant application to determine genomic markers of response and resistance to PD-L1 checkpoint inhibition in bladder cancer. I have proposed to do this by leveraging clinical samples obtained as part of a Phase II neoadjuvant clinical trial of atezolizumab in patients with localized bladder cancer unfit for or refusing chemotherapy. Working with colleagues at UCFS (Co-Is Quigley, Feng, and Fong) I have proposed to perform DNasequencing and neoepitope identification, RNasequencing to identify signatures predictive of response from tumor and tumor infiltrating lymphocytes, profiling of circulating tumor DNA, and to identify specific neoepitopes recognized by the immune system using tetramer experiments with matched, patient derived PBMCs.			
Principal investigator, lead coordinator for all Aims and for clinical trial.			
2. In review P0518659	Co investigator	3% % effort	Fong (PI)
CA160342			
US Department of Defense, Peer Reviewed Cancer Research Program, Team Science Award, W81XWH-16-PRCRP-TTSA		2017	

Identification of Somatic and Immunologic Predictors of
Response to Checkpoint Immune therapy in Bladder
Cancer

\$ 3M total

This team science proposal, authored by Dr. Friedlander in close conjunction with Dr. Fong, aims to identify mechanisms of response and resistance to PD-1 checkpoint immunotherapy, by acquiring and analyzing tissue and blood biospecimens from men participating in the Alliance Cooperative Group "STATURE" study of Atezolizumab in patients with metastatic platinum refractory bladder cancer. Comprehensive DNA, RNA, and cell free DNA profiling is proposed to identify mutational burdens, predicted neoepitopes, signatures of immune activation and signatures predictive of response to therapy, along with serial profiling of immune cell subsets and T cell receptor clonality.

Clinical lead coordinator among 5 sites (UCSF, UNC, UChicago, Weill-Cornell, and MSKCC).

3. In review	Co-investigator	5% % effort	Rider (PI)
NIH R21 CA217117-01			
Detection and underlying biology of prostate tumors in HIV-positive versus HIV- negative men			
<p>This R21 grant seeks to understand the discrepancy in prostate cancer (PCa) incidence between HIV+ and HIV- men. Differences in PSA screening and prostate tumor biology have been implicated as potential explanations, though neither has been sufficiently studied. This grant proposes to collect PCa clinical data from medical records of PCa cases in the Multicenter AIDS Cohort Study (MACS). This will enable us to accurately characterize prostate tumors by HIV status. Next, we hypothesize that among men who later develop PCa, age-specific PSA levels and PSA velocity are lower in HIV-positive men, rendering clinically utilized PSA thresholds less sensitive for PCa detection. To evaluate this hypothesis, we will use repeated blood samples collected from PCa cases and controls prior to diagnosis in the MACS cohort to measure total PSA concentrations and compare age-specific PSA levels by HIV status. We will also explore associations between PSA levels and indicators of HIV progression and determine whether the association between PSA levels and PCa development is modified by HIV status. Finally, we hypothesize that tumors arising in HIV-infected men differentially express genes related to PCa outcomes compared to tumors in HIV- uninfected men. Utilizing existing or already funded gene expression and linked clinical data from HIV-infected cases in the UroOnc Database (UODB) at the University of California-San Francisco and HIV-negative PCa cases from the Harvard Prostate Tumor Biorepository, we will conduct gene set enrichment analyses to compare specific gene pathways by HIV status. To facilitate larger, more comprehensive future follow-up studies we will develop an algorithm to accrue additional HIV-positive cases in the UODB. Completion of this study will illuminate differences in PCa detection and tumor biology according to HIV status. Results could also inform evidence-based PCa screening and management strategies in HIV-infected men, for whom there are currently no population-specific guidelines.</p>			
Clinical coordination of samples from UCSF UODB tissue bank. Clinical lead.			
4. In review	PI	10% % effort	Friedlander (PI)
University of California, Multicampus Research Programs 2017 and Initiatives (MRPI) Two Year Planning/Pilot Award			
Predicting Chemotherapy Resistance in Bladder Cancer			

This is a grant to establish a multi-center, UC wide infrastructure for collecting and analyzing pre- and post-cystectomy bladder cancer tissue as well as circulating tumor cells from patients undergoing neoadjuvant chemotherapy and cystectomy. It is hypothesized that a signature consisting of TCGA subtype, mutational profile, and presence of absence of circulating tumor cells can identify patients likely to respond or be resistant to neoadjuvant chemotherapy.

Lead coordinator for all sites, clinical lead. Coordination with laboratory investigators.

RESEARCH AWARDS - PAST

1. A114463	PI		Friedlander (PI)
American Society of Clinical Oncology, Young Investigator Award		7/1/2010	6/30/2011
Determination of Genotypic Markers of Docetaxel Resistance in Castration Resistant Prostate Cancer.		\$ 50,000 direct/yr 1	\$ 50,000 total
Young Investigator Award: the goal of this study is to identify the genetic changes associated with chemotherapy resistance that occur in men with prostate cancer treated with docetaxel chemotherapy. Role: PI			
2. A119352	PI	30 % effort	Friedlander (PI)
Prostate Cancer Foundation		03/01/2012	
Investigation of Genomic Mechanisms of Androgen Biosynthesis Inhibitor Resistance in Castration Resistant Prostate Cancer		\$ 75,000 direct/yr 1	\$ 225,000 total
Young Investigator Award Lead Investigator			
3. P0043122	PI	60 % effort	Friedlander (PI)
US Department of Defense		05/01/2012	04/30/2017
Investigation of Genomic Mechanisms of Androgen Biosynthesis Inhibitor Resistance in Castration Resistant Prostate Cancer		\$ 130,000 direct/yr 1	\$ 650,000 total
Physician Research Training Award, US Dept of Defense Lead Investigator			
4. NA	PI	5% % effort	Friedlander (PI)
Cancer League		8/1/2015	8/1/2017
Using Circulating Tumor Cells to Tailor Therapy for Patients with Metastatic Bladder Cancer		\$ 50,000 direct/yr 1	
This study seeks to isolate, purify, and culture circulating tumor cells from patients with bladder cancer in order to identify new drug targets for therapy.			
I serve as the clinical lead for this study, coordinating patient recruitment and acquisition of blood samples. I also advise the lab about the results presented at periodic lab meetings.			

PEER REVIEWED PUBLICATIONS

1. Friedlander TW, Weinberg VK, Small EJ, Sharib J, Harzstark AL, Lin AM, Fong L, Ryan CJ. Effect of the somatostatin analog octreotide acetate on circulating insulin-like growth factor-1 and related peptides in patients with non-metastatic castration-resistant prostate cancer: results of a phase II study. *Urol Oncol*. 2012 Jul-Aug; 30(4):408-14. PMID: 20884247
2. Friedlander TW, Weinberg VK, Huang Y, Mi JT, Formaker CG, Small EJ, Harzstark AL, Lin AM, Fong L, Ryan CJ. A phase II study of insulin-like growth factor receptor inhibition with nordihydroguaiaretic acid in men with non-metastatic hormone-sensitive prostate cancer. *Oncol Rep*. 2012 Jan; 27(1):3-9. PMID: 21971890
3. Friedlander TW, Roy R, Tomlins SA, Ngo VT, Kobayashi Y, Azameera A, Rubin MA, Pienta KJ, Chinnaiyan A, Ittmann MM, Ryan CJ, Paris PL. Common structural and epigenetic changes in the genome of castration-resistant prostate cancer. *Cancer Res*. 2012 Feb 1; 72(3):616-25. PMID: 22158653
4. Kim W, Zhang L, Wilton JH, Fetterly G, Mohler JL, Weinberg V, Morse A, Szmulewitz RZ, Friedlander TW, Fong L, Lin AM, Harzstark AL, Molina A, Small EJ, Ryan CJ. "Sequential Use of the Androgen Synthesis Inhibitors Ketoconazole and Abiraterone Acetate in Castration-Resistant Prostate Cancer and the Predictive Value of Circulating Androgens" *Clin Cancer Res*. 2014 Oct 21. [Epub ahead of print]
5. Friedlander TW, Ngo VT, Dong H, et al: Detection and characterization of invasive circulating tumor cells (ictcs) derived from men with metastatic castration resistant prostate cancer (mCRPC). *Int J Cancer*. 2014 May 15;134(10):2284-93. doi: 10.1002/ijc.28561. Epub 2014 Jan 2.
6. Kim W, Zhang L, Wilton JH, Fetterly G, Mohler JL, Weinberg V, Morse A, Szmulewitz RZ, Friedlander TW, Fong L, Lin AM, Harzstark AL, Molina A, Small EJ, Ryan CJ. Sequential use of the androgen synthesis inhibitors ketoconazole and abiraterone acetate in castration-resistant prostate cancer and the predictive value of circulating androgens. *Clin Cancer Res*. 2014 Dec 15; 20(24):6269-76. PMID: 25336698. PMCID: PMC4277885
7. Alva A, Friedlander T, Clark M, Huebner T, Daignault S, Hussain M, Lee C, Hafez K, Hollenbeck B, Weizer A, Premasekharan G, Tran T, Fu C, Ionescu-Zanetti C, Schwartz M, Fan A, Paris P. Circulating Tumor Cells as Potential Biomarkers in Bladder Cancer. *J Urol*. 2015 Sep; 194(3):790-8. PMID: 25912492. PMCID: PMC5103704
8. Premasekharan G, Gilbert E, Okimoto RA, Hamirani A, Lindquist KJ, Ngo VT, Roy R, Hough J, Edwards M, Paz R, Foye A, Sood R, Copren KA, Gubens M, Small EJ, Bivona TG, Collisson EA, Friedlander TW, Paris PL. An improved CTC isolation scheme for pairing with downstream genomics: Demonstrating clinical utility in metastatic prostate, lung and pancreatic cancer. *Cancer Lett*. 2016 Sep 28; 380(1):144-52. PMID: 27343980
9. Wei XX, Chan S, Kwek S, Lewis J, Dao V, Zhang L, Cooperberg MR, Ryan CJ, Lin AM, Friedlander TW, Rini B, Kane C, Simko JP, Carroll PR, Small EJ, Fong L. Systemic GM-CSF Recruits Effector T Cells into the Tumor Microenvironment in Localized Prostate Cancer. *Cancer Immunol Res*. 2016 Sep 29. PMID: 27688020. PMCID: PMC5115633
10. Clark PE, Spiess PE, Agarwal N, Bangs R, Boorjian SA, Buyyounouski MK, Efsthathiou JA, Flaig TW, Friedlander T, Greenberg RE, Guru KA, Hahn N, Herr HW, Hoimes C, Inman BA, Kader AK, Kibel AS, Kuzel TM, Lele SM, Meeks JJ, Michalski J, Montgomery JS, Pagliaro LC, Pal SK, Patterson A, Petrylak D, Plimack ER, Pohar KS, Porter MP, Sexton

WJ, Siefker-Radtke AO, Sonpavde G, Tward J, Wile G, Dwyer MA, Smith C. NCCN Guidelines Insights: Bladder Cancer, Version 2.2016. *J Natl Compr Canc Netw*. 2016 Oct; 14(10):1213-1224. PMID: 27697976

11. Anantharaman A, Friedlander T (co-first author), Lu D, Krupa R, Premasekharan G, Hough J, Edwards M, Paz R, Lindquist K, Graf R, Jendrisak A, Louw J, Dugan L, Baird S, Wang Y, Dittamore R, Paris PL. Programmed death-ligand 1 (PD-L1) characterization of circulating tumor cells (CTCs) in muscle invasive and metastatic bladder cancer patients. *BMC Cancer*. 2016 Sep 22;16(1):744. PubMed PMID: 27658492; PubMed Central PMCID: PMC5034508.
12. Friedlander TW, Graff JN, Zejnullahu K, Anantharaman A, Zhang L, Paz R, Premasekharan G, Russell C, Huang Y, Kim W, Aggarwal RR, Lin AM, Fong L, Alumkal JJ, Beer TM, Sharifi N, Alyamani M, Dittamore R, Small EJ, Paris PL, Ryan CJ. High-Dose Abiraterone Acetate in Men With Castration Resistant Prostate Cancer. *Clin Genitourin Cancer*. 2017 Jun 03. PMID: 28655452
13. Friedlander TW, Pritchard CC, Beltran H. Personalizing Therapy for Metastatic Prostate Cancer: The Role of Solid and Liquid Tumor Biopsies. *Am Soc Clin Oncol Educ Book*. 2017; 37:358-369. PMID: 28561699
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15. Friedlander TW, Graff JN, Zejnullahu K, Anantharaman A, Zhang L, Paz R, Premasekharan G, Russell C, Huang Y, Kim W, Aggarwal RR, Lin AM, Fong L, Alumkal JJ, Beer TM, Sharifi N, Alyamani M, Dittamore R, Small EJ, Paris PL, Ryan CJ. High-Dose Abiraterone Acetate in Men With Castration Resistant Prostate Cancer. *Clin Genitourin Cancer*. 2017 Jun 03. PMID: 28655452
16. Aggarwal R, Bryce A, Ryan CJ, Harzstark A, Derleth C, Kim W, Friedlander T, Lin AM, Rodvelt-Bagchi T, Dhawan M, Zhang L, Lee M, Siebeneck E, Hough J, Small EJ. A multicenter phase I study of cabazitaxel, mitoxantrone, and prednisone for chemotherapy-naïve patients with metastatic castration-resistant prostate cancer: A department of defense prostate cancer clinical trials consortium study. *Urol Oncol*. 2017 Feb 01. PMID: 28161323
17. Wei XX, Hsieh AC, Kim W, Friedlander T, Lin AM, Louttit M, Ryan CJ. A Phase I Study of Abiraterone Acetate Combined with BEZ235, a Dual PI3K/mTOR Inhibitor, in Metastatic Castration Resistant Prostate Cancer. *Oncologist*. 2017 Mar 17. PMID: 28314838. PMCID: PMC5423513

REVIEW ARTICLES

1. Anantharaman A, Friedlander T. "Targeting the Androgen Receptor in Metastatic Castrate-Resistant Prostate Cancer: A Review" *Urol Oncol*. 2015 Dec 16.
2. Friedlander TW, Premasekharan G, Paris PL. Looking back, to the future of circulating tumor cells. *Pharmacol Ther*. 2014 Jun;142(3):271-80. doi: 10.1016/j.pharmthera.2013.12.011. Review. PubMed PMID: 24362084.

3. Friedlander TW, Premasekharan G, Paris PL (2014). Looking Back, to The Future of Circulating Tumor Cells". *Pharmacol Ther*; 142(3): 271-280
4. Friedlander TW, Ryan CJ. "Targeting the Androgen Receptor" *Urol Clin N Am* 2012 Nov (39): 453-464
5. Friedlander TW, Ryan CJ. Novel hormonal approaches in prostate cancer. *Curr Oncol Rep* 2009; 11:227-234

BOOKS AND CHAPTERS

1. Friedlander TW, Ryan CJ, Small EJ, Torti F "Testicular Cancer" in Niederhuber, Armitage, Doroshow, Kastan, Tepper (Eds) *Abeloff's Clinical Oncology*, 5th Edition. Elsevier (Churchill Livingstone)
2. Friedlander, T.W. Ryan, C.J. (2010). □Andrenal Androgen Synthesis Inhibitor Therapies in Castration Resistant Prostate Cancer. □ In W. Figg, E. Small, C. Chau (Eds.), *Drug Management of Prostate Cancer*. (pp 91-100). Totowa: Humana Press.

OTHER PUBLICATIONS

1. Anantharaman A, Friedlander TW. A Stepping Stone Toward Personalized Oncology: Genomic Analysis of Circulating Tumor Cells to Guide Management of Metastatic Castration-resistant Prostate Cancer. *Eur Urol*. 2015 Aug 13. pii: S0302-2838(15)00712-5.
2. Friedlander TW, Fong L. (2014). The End of the Beginning: Circulating Tumor Cells as Prognostic Biomarkers in Castration Resistant Prostate Cancer. (Editorial) *J Clin Oncol*;32(11):1104-6
3. Friedlander TW. (2013) Whole Exome Sequencing to Map the Genomic Evolution of Metastatic Prostate Cancer. (Editorial) *Hum Mutat* 34:1231-1241
4. Friedlander TW, Ryan CJ. (2010) Editorial Comment on Adrenocorticotrophic hormone (ACTH) regulates androgen synthesis in men receiving androgen deprivation therapy for localized prostate cancer. *J Urol*;184(5):1976
5. Friedlander, TW; Ryan CJ. □Beyond the Abstract □ Functional phenotyping and genotyping of circulating tumor cells from patients with castration resistant prostate cancer. □ *UroToday* 7 Aug. 2009. Available from: http://www.urotoday.com/3341/browse_categories/beyond_the_abstract/beyond_the_abstract_functional_phenotyping_and_genotyping_of_circulating_tumor_cells_from_patients_with_castration_resistant_prostate_cancer08072009.html

SIGNIFICANT PUBLICATIONS

1. See "Peer Reviewed Publications" above

CONFERENCE ABSTRACTS

1. See "Invited Presentations"
2. Phase I study of pazopanib (PAZ) in combination with abexinostat (ABX) in patients with metastatic solid tumors. ASCO Annual Meeting

3. Terence W. Friedlander, Vivian K. Weinberg, Alex Yeung, James Burke, Donald L. Lamm, James M. McKiernan, John J. Nemunaitis, Joe Stephenson Jr., Eric Jay Small, Lawrence Fong, Maxwell V. Meng; **Activity of intravesical CG0070 in rb-inactive superficial bladder cancer after BCG failure: Updated results of a phase I/II trial.**
Presented at ASCO Genitourinary Symposium and ASCO Annual Meeting 2012

OTHER CREATIVE ACTIVITIES

1. 2014 M3 Oncology Module (2nd year medical students) prostate cancer case study and question guide.

UCSF Mini-Medical school videotaped lectures about Bladder Cancer and Testicular Cancer, available online.: over 75,000 views on YouTube as of 2016

Prostate Cancer Treatment and Research Handout for patients in GU Medical Oncology clinic, describing how prostate cancer is treated and describing current UCSF research

ADDITIONAL RELEVANT INFORMATION

Fluent in Spanish, conversant in French and Italian

High-Dose Abiraterone Acetate in Men With Castration Resistant Prostate Cancer

Terence W. Friedlander,¹ Julie N. Graff,² Kreshnik Zejnullahu,³
 Archana Anantharaman,¹ Li Zhang,⁴ Rosa Paz,¹ Gayatri Premasekharan,⁵
 Carly Russell,¹ Yong Huang,⁶ Won Kim,¹ Rahul R. Aggarwal,¹ Amy M. Lin,¹
 Lawrence Fong,¹ Joshi J. Alumkal,² Tomasz M. Beer,² Nima Sharifi,⁷
 Mohammad Alyamani,⁸ Ryan Dittamore,⁹ Eric J. Small,¹
 Pamela L. Paris,^{1,5} Charles J. Ryan¹

Abstract

Abiraterone acetate with prednisone prolongs progression-free and overall survival in men with advanced prostate cancer, but most eventually acquire resistance to treatment. In this study we evaluated the clinical benefit of increasing the dose of abiraterone acetate in patients who develop acquired resistance to standard-dose therapy while exploring the pharmacokinetics and pharmacodynamics of resistance.

Background: Abiraterone acetate (AA) inhibits androgen biosynthesis and prolongs survival in men with metastatic castration-resistant prostate cancer (mCRPC) when combined with prednisone (P). Resistance to therapy remains incompletely understood. In this open-label, single-arm, multicenter phase II study we investigated the clinical benefit of increasing the dose of AA at the time of resistance to standard-dose therapy. **Patients and Methods:** Eligible patients had progressive mCRPC and started AA 1000 mg daily and P 5 mg twice daily. Patients who achieved any prostate-specific antigen (PSA) decline after 12 weeks of therapy continued AA with P until PSA or radiographic progression. At progression, AA was increased to 1000 mg twice daily with unchanged P dosing. Patients were monitored for response to therapy for a minimum of 12 weeks or until PSA or radiographic progression. The primary end point was PSA decline of at least 30% after 12 weeks of therapy at the increased dose of AA. **Results:** Forty-one patients were enrolled from March 2013 through March 2014. Thirteen men experienced disease progression during standard-dose therapy and were subsequently treated with AA 1000 mg twice per day. Therapy was well tolerated. No PSA declines $\geq 30\%$ nor radiographic responses were observed after 12 weeks of dose-escalated therapy. Higher baseline dehydroepiandrosterone levels, lower circulating tumor cell burden, and higher pharmacokinetic levels of abiraterone and abiraterone metabolites were associated with response to standard-dose therapy. **Conclusion:** Increasing the dose of abiraterone at the time of resistance has limited clinical utility and cannot be recommended. Lower baseline circulating androgen levels and interpatient pharmacokinetic variance appear to be associated with primary resistance to AA with P.

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Keywords: Acquired resistance, Androgen receptor, Androgens, Hormonal therapy, Primary resistance

This trial is registered at [Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01637402): NCT01637402.

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High-Dose AA in Men With CRPC

Introduction

Most castration-resistant prostate cancer (CRPC) tumors remain androgen-dependent despite castrate levels of testosterone.¹ The mechanisms by which CRPC tumors grow in the castrate environment are incompletely understood but are hypothesized to be a result of increased intratumoral androgen synthesis, androgen receptor (*AR*) gene amplification or overexpression, *AR* mutations that increase affinity for low potency androgens, and constitutively active *AR* gene splice variants.²

Studies have consistently shown that intratumoral androgen levels are increased in the CRPC microenvironment although this might not be reflected using conventional serum androgen assays.² Abiraterone acetate (AA) is a potent inhibitor of androgen synthesis, and has been shown in combination with prednisone (P) to be an effective treatment modality in men with metastatic CRPC (mCRPC) in the pre- as well as postchemotherapy settings.^{3,4} Despite pharmacokinetic studies showing increases in the abiraterone area under the curve as well as maximum concentration for doses of 2000 mg per day without increased dose-limiting toxicities, a dose of 1000 mg daily was selected for development after phase I studies showed maximal androgen suppression with this dosage.⁵ Interestingly, large interpatient pharmacokinetic variability was notable for patients taking AA 1000 mg daily in the initial studies, and it remains unclear whether these observed differences have clinical implications.⁵

Circulating androgen levels are known to increase at the time of clinical progression during treatment with other inhibitors of androgen synthesis such as ketoconazole, a less potent inhibitor of androgen synthesis.⁶ We hypothesize that increasing the dose of AA at the time of disease progression during standard-dose therapy would increase its clinical effects, thereby further reducing androgen synthesis, slowing tumor growth, and resulting in second responses to therapy.

In this article we report the results of a phase II study on the efficacy and safety of dose-escalated AA (2000 mg/d) in combination with P (10 mg/d) for patients who experience disease progression after an initial response to standard-dose AA (1000 mg/d) in combination with P (10 mg/d). We aim to describe the clinical efficacy, safety, pharmacokinetics, and changes in circulating tumor cell (CTC) burden for each dose of AA in combination with P.

Patients and Methods

Study Design

This was an investigator-initiated, single-arm, open-label, multicenter phase II study of standard-dose AA (1000 mg daily) in combination with P (5 mg twice daily) followed by dose-escalated AA (1000 mg twice daily) in combination with P (5 mg twice daily) at the time of disease progression in patients with chemotherapy-naïve, mCRPC (see [Supplemental Figure 1](#) in the online version). The primary study end point was a prostate-specific antigen (PSA) decline of at least 30% after 12 weeks of increased-dose therapy, as per Prostate Cancer Working Group 2 (PCWG2) criteria.⁷ Secondary objectives were safety and tolerability of increased-dose AA. Correlative analyses were used to evaluate changes in androgen levels, pharmacokinetics, and CTC burden.

All eligible patients received a baseline complete physical examination, laboratory evaluation including complete blood count,

serum renal and liver function tests (LFTs), serum PSA, serum androgens including dehydroepiandrosterone (DHEA), androstenedione, and testosterone. Baseline imaging using computer tomography or magnetic resonance imaging of the abdomen and pelvis, and radionuclide bone imaging were obtained before enrollment and repeated every 12 weeks thereafter. All enrolled patients were followed every 4 weeks (1 cycle = 4 weeks) with physical examinations, and laboratory and radiographic testing, as appropriate until discontinuation from trial. Abiraterone levels were measured at the beginning of cycle 2, at the time of initial disease progression during standard-dose therapy, and at 2 and 4 weeks after the start of increased-dose therapy.

Patient Population

Eligible patients had progressive mCRPC per PCWG2.⁷ No previous AA, enzalutamide, ketoconazole, or chemotherapy was allowed. The study was approved by the University of California, San Francisco (UCSF) and Oregon Health & Science University institutional review boards, all patients provided written informed consent before participation, and the trial was registered with the clinicaltrials.gov identifier NCT01637402.

Study Therapy

All patients started therapy with AA 1000 mg daily and P 5 mg twice daily (see [Supplemental Figure 1](#) in the online version). AA was taken on empty stomach at least 2 hours after or 1 hour before a meal. Dosing was interrupted for Grade ≥ 3 toxicities related to study treatment and was restarted at a 25% reduced dose when toxicities resolved to Grade ≤ 1 . Patients who achieved any PSA decline after 3 cycles (12 weeks) of therapy continued to receive AA with P. Patients who did not exhibit a PSA decline after 3 cycles of therapy were deemed “primary-resistant,” and were removed from the study. Continuing patients received standard-dose AA with P until PSA increase of 25% above nadir or clinical or radiographic progression, per consensus criteria.⁷ At the time of disease progression, the AA dose was increased to 1000 mg twice daily, P was maintained at 5 mg twice daily, and patients were monitored for a minimum of 3 cycles (12 weeks) or until subsequent PSA, radiographic, or clinical progression, per PCWG2 criteria, which was the standard of care at the time.

Abiraterone Pharmacokinetics

All enrolled patients had pharmacokinetic measurement of plasma abiraterone levels using liquid chromatography coupled with tandem mass spectrometry performed at the UCSF Drug Studies Unit. Patients were instructed not to take their assigned AA dose the morning of the laboratory appointment and presented for phlebotomy between 8 am and 12 pm on the morning of the first day of therapy, the first day of cycle 2 (4 weeks after the start of treatment), and at the time of disease progression during standard-dose therapy. This was repeated at 2 and 4 weeks after the start of dose-escalated therapy. The assay was validated from 0.1 ng/mL to 50 ng/mL with accuracy (percentage of deviation) and precision (percentage of coefficient of variation) within 15%. Abiraterone metabolites were detected using a validated liquid chromatography - mass spectrometry method at Cleveland Clinic that distinguished these steroidal products, many of which are diastereoisomers.⁸

Circulating Tumor Cells

Circulating tumor cells were collected using Vita-Assay plates (Vitalex), which enrich for cells able to invade into a collagenous, fluorescently-labeled cell adhesion matrix (CAM).⁹ Nucleated cells from 40-mL patient blood samples were pelleted using centrifugation at 1000 rpm for 5 minutes after red blood cell (RBC) lysis was performed twice with RBC lysis buffer. The cells were resuspended and plated with Complete Cell Culture (CCC) media (American Type Culture Collection, Manassas, VA) (1:1 mixture of Dulbecco's modified Eagle's medium and Roswell Park Memorial Institute 1640 medium supplemented with 10% calf serum, 10% Nu-serum, 2 mM L-glutamine, 1 unit/mL penicillin, and 10 µg/mL streptomycin) for 24 hours using a Vita-Assay AG6W (Alexa 488-CAM coated) plate (Vitalex) at 37°C. After overnight incubation, nonadherent cells were removed and fresh CCC media was added to the plate. CAM-positive cells were then released by adding the cell-releasing CAM enzyme (Vitalex) onto the plate. The cells were prepared for fluorescence activated cell sorting using an ARIA III (BD Biosciences) by washing with phosphate buffered saline with 0.2% bovine serum albumin buffer and staining for 30 minutes at room temperature with allophycocyanin anti-CD45/CD14 antibodies. Excess noncellular particles were filtered out through a cell-strainer cap (Becton Dickinson). Invasive CTCs were identified as CAM^{high}/CD45/14^{low}/4',6-diamidino-2-phenylindole, dihydrochloride^{high} and sorted into 0.2-mL polymerase chain reaction tubes (10-cell aliquots). Expression of CD44, a putative marker of stemness, was also assessed in CTC samples. Cell counts were done using FlowJo software version 10.0 (Tree Star, Inc).¹⁰ A small subset of patient samples were sent to Epic Sciences (San Diego, CA) for CTC analysis.¹¹

Statistical Considerations

A Simon 2-stage minimax design was used for accrual to test for a 20% (null) versus 40% (alternative) proportion of patients

achieving a $\geq 30\%$ PSA decline from baseline after 12 weeks of treatment with AA 1000 mg twice daily with P 5 mg twice daily. To account for potential drop-out and 15% to 20% primary-resistant patients, 41 patients were required to achieve 33 patients who would be eligible to participate in the dose-escalated phase of the study, with a directional level of significance of 0.05 and power of 0.80. Interim analysis for efficacy was performed after 18 patients completed 3 cycles (12 weeks) of treatment with dose-escalated AA with P. Demographic and clinical characteristics were summarized using descriptive statistics. Frequency distributions and percentages were used to summarize categorical variables, and medians with ranges were used to describe continuous variables. Comparisons of continuous variables among groups were assessed using the 2-sample *t* test for 2 groups, and analysis of variance if more than 2 groups, when the normality assumption held. Otherwise the corresponding nonparametric equivalents, the Wilcoxon rank sum test and the Kruskal–Wallis test, were used. When comparing continuous variables between baseline and progression, the paired *t* test (or Wilcoxon test) was used. The statistical significance was declared at $\alpha < 0.05$ and no multiple testing adjustments were performed. All statistical analyses were done using the statistical computing software R (<https://www.r-project.org>).

Results

Patient Characteristics

Forty-one patients with mCRPC were enrolled in the study from March 2013 to March 2014 (Table 1). The median age of the study population was 68 years (range, 55–79 years). Twelve patients (29%) had a previous radical prostatectomy, 15 patients (37%) had previous definitive radiotherapy to the prostate, and 14 patients (34%) had never received definitive therapy to the prostate. Seventeen patients (41%) had bone-confined metastatic disease, 7 patients (18%) had lymph node-confined metastatic disease, and 17 patients (41%) had a combination of bone and lymph node metastatic disease at the time of study enrollment. The median baseline PSA, hemoglobin, lactate dehydrogenase (LDH) and alkaline phosphatase values are listed in Table 1.

Patient Disposition and Response to Standard Dose Therapy

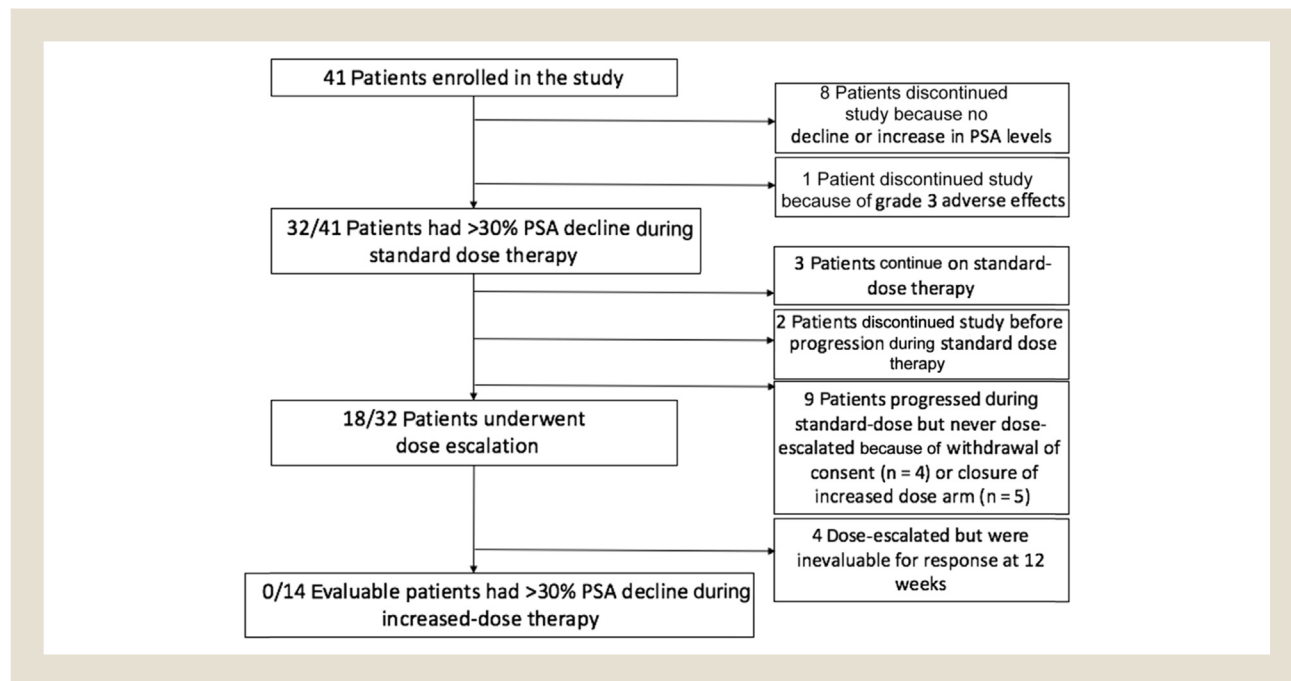
Thirty-two of 41 (78%) patients enrolled in the study achieved a decline in PSA levels from baseline after 12 weeks of standard-dose AA (1000 mg daily) with P (10 mg daily) therapy (Figure 1 and Figure 2A). Eight patients (19%) did not exhibit any PSA decline during AA with P treatment at 12 weeks of therapy and were deemed to have primary resistance to AA with P. These patients were removed from the study. One patient (2%) withdrew from the study before completing 3 cycles (12 weeks) of standard-dose therapy because of Grade 3 transaminitis, as well as pancreatitis and small bowel obstruction and was not considered evaluable for efficacy. The median time of standard-dose therapy among patients with primary resistance was 12 weeks (range, 8–12 weeks) and 53 weeks (range, 24–151) for responders (see Supplemental Figure 2 in the online). Despite PSA declines after the initial 12 weeks of therapy, 2 patients (5%) discontinued treatment before disease progression; 1 patient developed an unrelated other malignancy, and the second patient withdrew consent because of worsening hip pain.

Table 1 Patient Characteristics at the Time of Enrollment

Characteristic	Value
Patients, n (%)	41 (100)
Median Age (Range), y	68 (55–79)
Ethnicity, n (%)	
Caucasian	30 (73)
Asian	3 (7)
Unknown	8 (20)
Median Prostate-Specific Antigen (Range), ng/mL	23.18 (0.4–1763.9)
Median Hemoglobin (Range), g/dL	12.9 (9.9–14.7)
Median Lactate Dehydrogenase (Range), U/L	195 (109–450)
Median Alkaline Phosphatase (Range), U/L	81 (43–601)
Extent of Disease, n (%)	
Bone only	17 (41)
Lymph node only	7 (18)
Bone and lymph node	17 (41)
Previous Therapies, n (%)	
Radical prostatectomy	12 (29)
Definitive radiotherapy	15 (37)
Neither (metastatic at diagnosis)	14 (34)

High-Dose AA in Men With CRPC

Figure 1 Disposition to Therapy. Flow Chart of Enrollment and Patient Evaluation in Relation to Study Schema



Abbreviation: PSA = prostate-specific antigen.

Three patients (7.1%) continued to receive standard-dose therapy at the time of analysis.

Response to Dose-Escalated Abiraterone

A total of 32 patients had PSA declines with standard dose AA with P and were considered evaluable for response. Because of the 2-stage design, an interim analysis was performed after the first 18 of

these patients experienced disease progression during standard-dose AA with P and moved on to dose-escalation, receiving AA 1000 mg twice daily with P 5 mg twice daily in the higher-dose cohort. Four patients withdrew consent before completing 3 cycles (12 weeks) of dose-escalated AA with P, rendering them unevaluable for response. Although transient PSA declines were observed, 0 of 14 patients met the primary end point of achieving a 30% PSA decline with the

Figure 2 Waterfall Plot of Best Prostate-Specific Antigen (PSA) Responses for Evaluable Patients After 12 Weeks of Daily (A) Standard Dose Abiraterone Acetate (1000 mg) and Prednisone (10 mg) and (B) Dose-Escalated Abiraterone Acetate (1000 mg Twice Daily) and Prednisone (10 mg Daily). Each Bar Represents a Unique Patient. Y Scale Capped at 100% PSA Change. Dotted Line Indicates 30% Decline From Baseline PSA

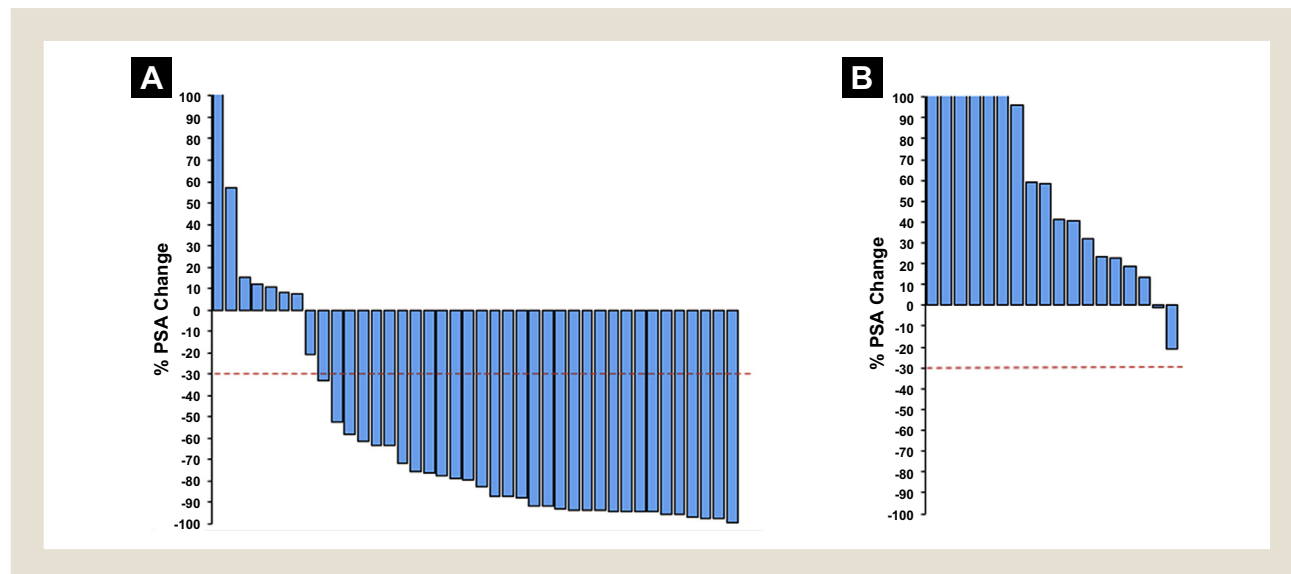
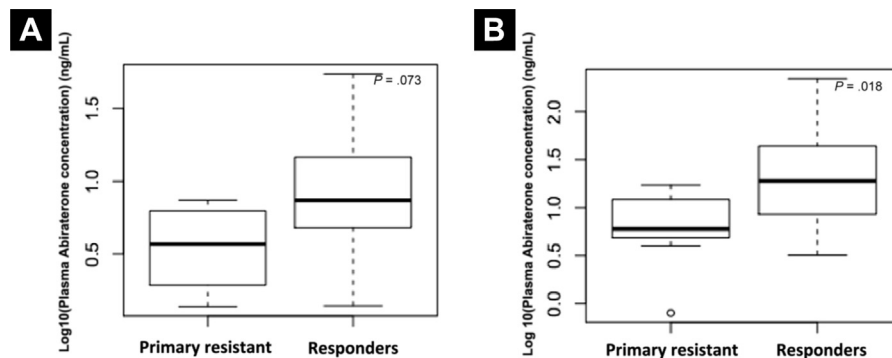


Figure 3 Steady State Plasma Abiraterone Acetate (AA) Concentrations in Primary-Resistant Compared With Responders to Standard-Dose Abiraterone Acetate and Prednisone Therapy After (A) 28 Days of Therapy and at (B) Prostate-Specific Antigen Progression. *P* Values Evaluate Difference in Plasma AA Concentrations Between Primary-Resistant and Responders at Indicated Time Points



increased dose of AA after 3 cycles (12 weeks) of therapy (Figure 1 and Figure 2B). Therefore, after this interim analysis for efficacy, further accrual to the dose-increased arm was halted. The median time of increased-dose therapy for all dose-escalated patients was 12 weeks (range, 6-14.5; see Supplemental Figure 2 in the online). Responses to subsequent therapies were not recorded prospectively.

Abiraterone Acetate Pharmacokinetics

Twenty-six patients (63%) taking AA 1000 mg with P 10 mg daily had baseline and week 4 pharmacokinetic samples available for measurement of abiraterone plasma concentrations. Seven samples were from patients with primary-resistant disease and 19 were from patients who had achieved a PSA decline during standard-dose therapy. The plasma abiraterone levels after 4 weeks of standard-dose therapy were lower among patients with primary-resistant disease compared with patients who achieved any PSA decline (median 3.69 ng/mL vs. 7.38 ng/mL; *P* = .073; Figure 3A). At the time of disease progression during standard-dose therapy, the plasma levels of abiraterone were significantly lower among patients with primary-resistant disease compared with responders (median, 6.00 ng/mL vs. 19.00 ng/mL; *P* = .018; Figure 3B).

Eight of 14 evaluable patients who received dose-increased AA had corresponding increases in the serum level of abiraterone compared with the standard dose; plasma abiraterone concentrations did not increase in the remaining 5 patients. Overall, a nonsignificant increase in median abiraterone levels was observed in patients taking the increased dose, from 14.2 ng/mL (range, 0.80-221) at the time of disease progression with the 1000-mg daily dose to 31.5 ng/mL (range, 5.2-148) with the 1000-mg twice-daily dose.

Levels of AA metabolites (D4A, 3-keto-5 α -Abi, 3 α -OH-5 α -Abi, 3 β -OH-5 α -Abi, 3-keto-5 β -Abi, 3 α -OH-5 β -Abi, and 3 β -OH-5 β -Abi)^{12,13} were measured in 26 patients at baseline and again at time of disease progression. Compared with patients who had a PSA decline, patients who had primary resistance to AA 1000 mg with P had lower baseline 3-keto-5 α -Abi (1.28 vs. 3.58 ng/mL; *P* = .025)

and 3 β -OH-5 α -Abi (median 0.14 vs. 0.20 ng/mL; *P* = .034) levels. Interestingly, in a comparison of patients with primary resistance, early progression (< 6-month response to therapy), and late progression (> 6-month response to therapy), baseline 3-keto-5 α -Abi (median 1.28, 5.36, and 3.49 ng/mL respectively; *P* = .042) and 3 β -OH-5 α -Abi (median, 0.14, 1.02, and 0.19 ng/mL, respectively; *P* = .039) levels remained statistically different between the 3 groups of patients.

Abiraterone Pharmacodynamics

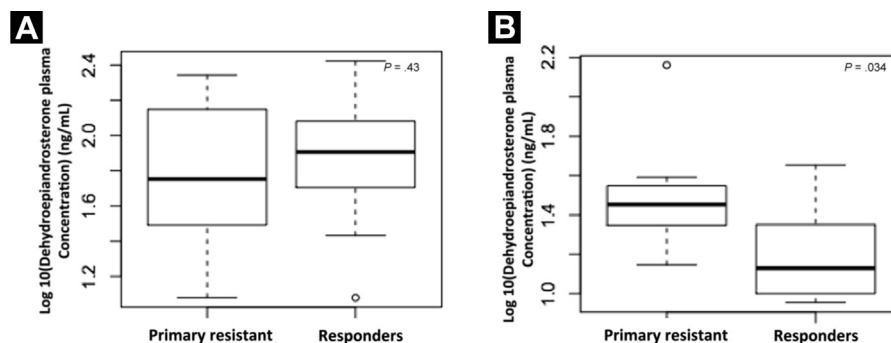
A significant decline in plasma DHEA levels was observed for all patients during the study. However, patients who had primary resistance to AA with P had significantly higher DHEA levels at the time of disease progression compared with patients whose disease progressed during AA with P treatment after initial PSA decline (28.50 vs. 13.50 ng/mL; *P* = .034; Figure 4B). Androstenedione as well as DHT were maximally suppressed below the limit of detection at the time of disease progression during standard-dose therapy. Therefore, evaluation of these hormonal changes with dose-escalated AA was not pursued.

Safety

A total of 39 adverse events were observed in patients who received standard-dose therapy. Only 1 significant Grade 3 and 1 significant Grade 4 event was observed in patients who received standard-dose AA with P. One patient experienced a Grade 3 episode of pancreatitis, transaminitis, nausea, and small bowel obstruction, thought due to the passage of a gallstone unrelated to study therapy. There was a single Grade 4 event (stroke) in a patient taking AA 1000 mg daily with P 10 mg daily, also thought to be unrelated to study therapy. Only 1 Grade 3 event (5.5%) of transaminitis was noted in patients receiving dose-escalated AA. This was attributed to increased dosing of the drug and resolved after a dose reduction of AA to 750 mg twice daily. The most common Grade 1 adverse events in patients taking standard-dose AA were hypertension (n = 5; 12%), elevated creatinine level

High-Dose AA in Men With CRPC

Figure 4 Plasma Dehydroepiandrosterone (DHEA) Concentrations in Patients With Primary Resistance to Standard-Dose Abiraterone Acetate and Prednisone Therapy Compared With Responders After (A) 28 Days of Therapy and at (B) Prostate-Specific Antigen Progression. *P* Values Evaluate Difference in Plasma DHEA Concentrations Between Primary-Resistant and Responders at Indicated Time Points



(*n* = 5; 12%), fatigue (*n* = 5; 12%), and increased LFTs (*n* = 3; 7.3% of patients; Table 2). Among the 18 patients receiving AA 1000 mg twice daily with P 10 mg daily, there were 2 (11%) Grade 1 adverse events (hypokalemia, dry mouth).

Circulating Tumor Cells

Of the 41 patients enrolled in the study, 40 (98%) were sampled for CAM-positive (CAM⁺)/CD45/14⁺ CTC collection with the VitaAssay at baseline before treatment. CTCs were detected in all analyzed samples. CTCs were collected for enumeration purposes

only. The assay for ARv7 splice variants was not available at the time of this study using this platform and was therefore not performed. The median number of CTCs identified was 26 CTCs/mL of blood (range, 1–423). The Wilcoxon test was performed to determine the association between baseline CTCs and the response to therapy. Response to therapy was divided into 3 groups for analysis: patients with no response (primary-resistant disease), responders whose disease progressed within 6 months of starting therapy (early progression), and responders whose disease progressed after 6 months of AA with P therapy (late progression).

Table 2 Adverse Events

Event	Standard-Dose Abiraterone Acetate		Increased Dose Abiraterone Acetate	
	Grade 1 or 2	Grade 3 or 4	Grade 1 or 2	Grade 3 or 4
Cystitis	1 (2.4)	—	—	—
Elevated Creatinine	5 (12.2)	—	—	—
Fatigue	5 (12.2)	—	—	—
Hot Flashes	2 (4.9)	—	—	—
Hypertension	5 (12.2)	2 (4.9)	—	—
Hypermagnesemia	1 (2.4)	—	—	—
Hypocalcemia	1 (2.4)	—	—	—
Hypokalemia	1 (2.4)	—	1 (2.4)	—
Hypomagnesemia	1 (2.4)	—	—	—
Hepatic Transaminitis	3 (7.3)	1 (2.4)	—	1 (2.4)
Pancreatitis	—	1 (2.4)	—	—
Memory Loss	1 (2.4)	—	—	—
Myalgias	2 (4.9)	—	—	—
Nausea	—	1 (2.4)	—	—
Palpitations	1 (2.4)	—	—	—
Rash	1 (2.4)	—	—	—
Shingles	1 (2.4)	—	—	—
Vascular Disorder	1 (2.4)	1 (2.4)	—	—
Weight Gain	1 (2.4)	—	—	—
Dry Mouth	—	—	1 (2.4)	—

Data are presented as *n* (%).

There was a nonsignificant trend toward shorter responses with standard-dose AA with P in patients with higher median CTC counts (41 vs. 16 CTCs/mL; $P = .177$) and higher CD44⁺ CTCs (19.5 vs. 8 CTCs/mL; $P = .214$). Patients with primary-resistant disease had higher median CTC counts (44 CTCs/mL) compared with patients with early progression (41 CTCs/mL) and late progression (16.7 CTCs/mL). CD44 is a putative marker of cancer stem cells¹⁴ and it was observed that patients with early progression had a higher median number of CD44⁺ CTCs (19.5 CTCs/mL) than those with late progression (8 CTCs/mL). Interestingly, an inverse pattern was observed in the relationship between baseline CTC clusters and response to therapy. Fewer CTC clusters were detected in primary-resistant patients compared with those with early or late progression, respectively (median 0.024 CTC clusters/mL vs. 0.073 CTC clusters vs. 0.133 CTCs/mL; $P = .085$). CTC clusters of epithelial origin were also observed in a subset of patient samples ($n = 16$) that were evaluated using the Epic CTC platform, although statistical inferences were not performed because of the limited sample size.

Discussion

This study was designed to investigate the clinical implications of maximizing androgen suppression by increasing the dose of AA from 1000 mg daily to 1000 mg twice daily at the time of disease progression. Although this study confirmed that treatment with AA 1000 mg twice daily in combination with P 5 mg twice daily is well tolerated, the increased AA dosing does not appear to yield any clinical benefit in those who acquire resistance to therapy. PSA changes according to PCWG2 criteria were used as the primary outcome measure to define disease progression in this study because they are easily measured and generally reflect AR activity. The pharmacodynamics and pharmacokinetic analyses performed as correlatives, however, give some insight into mechanisms of primary and acquired resistance to AA with P, as well as the lack of response to higher doses of AA with standard-dose P.

It has previously been shown that serum androgen levels are elevated at the time of resistance to ketoconazole, a less potent androgen synthesis inhibitor,^{10,15} implicating persistent androgen signaling as a driver of resistance. Despite this observation, in this study circulating testosterone, dihydrotestosterone (DHT), DHEA, and androstenedione were maximally suppressed at the time of resistance (Figure 4), therefore persistent serum androgens were unlikely to be driving resistance. Other AR-driven mechanisms of resistance include elevated intratumoral androgen concentrations, AR amplification, AR binding domain mutations, or altered AR transcriptional splicing. Intratumoral androgen levels might be higher than in circulation, however, this was not assessed in this study.¹⁶ Similarly, AR amplification^{17,18} has been hypothesized to increase sensitivity to minimal residual androgens and AR point mutations are thought to increase the binding affinity of androgens.¹⁹ Assessing these variables at the time of resistance to standard-dose AA in combination with P would allow for a better understanding of this process. Evaluating the benefit of cotargeting the AR with a combination of AA and a direct inhibitor of the androgen receptor, such as enzalutamide or apalutamide, is an active area of current research.²

Androgen-independent pathways of resistance might also develop, which allow for persistence of AR signaling in a ligand-independent fashion. Recently, specific point mutations in AR conferring resistance to AA with P (H874Y and T877A) while constitutively activating AR were described.²⁰ Mutations at the N-terminal domain or in the DNA binding domain of the AR gene have also been described, which alter the binding specificity of coregulators, consequently increasing the transcriptional activation of downstream genes allowing for persistence of the AR pathway.²¹⁻²⁴ Alternative splicing of the AR mRNA might lead to expression of constitutively active AR variants.²⁵ Several AR splice variants have been described, of these, AR-V7 has been well investigated and shown to predict de novo resistance to inhibitors of androgen synthesis and enzalutamide, a potent AR antagonist.²⁶ AR-independent pathways leading to transcription of AR-dependent genes have also been described.²⁷ Activation of the glucocorticoid receptor has been shown to result in potent activation of AR-responsive genes in experimental models of resistance to treatment with AA and P and in patient samples.²⁸ Last, the differentiation of prostate adenocarcinoma to a small cell/neuroendocrine phenotype has been shown to result in resistance to antiandrogen therapies and might contribute to AA and P therapy resistance.¹⁵ Although these factors were not specifically evaluated in this study, all could contribute to either primary or acquired resistance to AA with P.

One of the more intriguing findings from this study was the observation that patients with primary-resistant disease to standard-dose AA with P had lower abiraterone concentrations after 4 weeks of therapy and at the time of disease progression. Although observed variations in abiraterone levels could result from several factors, including chance observation because of the limited number of patients and differences in timing of plasma abiraterone measurements, it is possible that there are genetic predispositions among patients that lead to differential abiraterone metabolism, and this warrants investigation in further studies. Alternatively, drug-drug interactions might alter the metabolism of AA and lead to decreased plasma abiraterone levels. Review of concomitant medications revealed no consistent pattern of interactions, making drug-drug interactions an unlikely explanation for this difference. Similarly, nonadherence is unlikely to explain the difference because patients were required to have drug diaries, which did not reveal any irregularities. Whether dose escalation would be of benefit to patients with primary refractory disease was not explored in this study because alternative therapies, such as chemotherapy, were available for these patients with aggressive disease. In light of these findings, a dedicated pharmacokinetic study with inpatient dose escalation in this population could be considered.

The findings of decreased abiraterone plasma concentrations in primary-resistant patients are intriguing because abiraterone pharmacokinetics have been the subject of recent interest.²⁵ AA is thought to be converted to its active agent, abiraterone, in hepatocytes. Conversion of AA to another active metabolite, termed D4A, has been determined to have more potent antitumor activity, whereas 3-keto-5 α -Abi, another downstream metabolite, has AR agonist activity. We observed that nonresponders had lower plasma abiraterone concentrations after 4 weeks of treatment and again at the time of PSA progression (Figure 3). However, baseline 3-keto-5 α -Abi and

High-Dose AA in Men With CRPC

3 β -OH-5 α -Abi metabolite levels were statistically higher in responders to AA 1000 mg with P compared with primary-resistant patients. There are at least 2 possible explanations. First, higher levels of these AA metabolites might simply be a consequence of higher levels of exposure to the parent drug. Second, the formation of these metabolites might reflect differences in endogenous steroidal metabolism among patients, which also affects AA metabolism in parallel. Analysis of levels at the time of disease progression did not show any statistically meaningful trends and there were not enough samples available to make a meaningful comparison of these levels in early versus late progressors. This suggests that although acquired resistance might not be pharmacokinetically driven, primary resistance might be, in part, because of either inadequate exposure to drug or to rapid metabolism leading to lower levels of active drug and drug metabolites. Although these findings need to be confirmed in a larger population, measurement of these metabolites might be a useful tool in predicting response to AA with P. Dose-escalated AA in this primary-resistant population might be meaningful and could be explored in future studies.

Similar patterns were observed in DHEA levels between non-responders and responders. DHEA is an endogenous steroid hormone produced by the adrenal glands and gonads, and is one of the most abundant circulating androgens.²⁹ High circulating DHEA levels in men with CRPC are thought to indicate an androgen-rich environment, and correlate with a higher likelihood of response to AA and P.³⁰ Primary-resistant patients had a trend toward higher DHEA levels at week 4 and significantly higher DHEA levels at the time of disease progression compared with those who responded to therapy (Figure 4). This is suggestive of continued androgen dependence in this population and might reflect underlying pharmacokinetic differences. Whether DHEA could be used to risk-stratify patients in future studies is worth exploration in a prospectively designed study, and similarly biopsy and interrogation of the AR axis could help better define the biology of tumors that progress in a low DHEA environment.

Circulating tumor cells are circulating biomarkers that can provide patient-specific prognostic and predictive information. A decline in CTCs in men with CRPC has been shown to be prognostic of outcome in response to chemotherapy in men with prostate cancer,³¹ and a composite score of decline in CTCs measured using the CellSearch system (Veridex) along with changes in serum LDH has been shown to fulfill criteria for surrogacy for survival.^{32,33} Similarly, the detection of *AR-V7* transcripts in CTCs has been shown to predict response to AA and P or enzalutamide.²⁶ In this study, CTCs were collected from patients using an assay to enrich for cells capable of invading a collagenous matrix. Patients with more treatment-resistant disease had a trend toward higher number of CTCs consistent with previous studies and had a higher frequency of expression of CD44, a putative marker for stemness. Interestingly, higher baseline CTC clusters were associated with a more favorable response to the drug contrary to other findings.³⁴⁻³⁶ Of note, CTC clusters of epithelial origin (CK⁺/CD45⁻) were observed in a subset of patient samples (n = 16) that were evaluated using the Epic CTC platform. Higher baseline CTC clusters might help identify patients with more epithelial-like tumors that should have a better response to AA with P. These observations require

further exploration in a larger, prospective study, but have the potential to be biomarkers predictive of response to AA with P.

Conclusion

Although well tolerated, increasing the dose of AA at the time of acquired resistance to standard dose therapy was not effective and cannot be recommended. The pharmacokinetic differences observed in primary-resistant patients and the observation of an association between DHEA levels, CTCs, and response should be further evaluated to determine if this could be an effective biomarker to predict resistance to abiraterone.

Clinical Practice Points

- Metastatic CRPC is the lethal form of the disease. Although AA, a potent and specific androgen synthesis inhibitor, in combination with P, prolongs progression-free and overall survival in this patient population, most patients eventually develop acquired resistance to treatment.
- Higher doses of AA have previously been shown to be well tolerated, however the clinical utility of increasing the dose is unknown.
- This study showed that there is limited clinical utility in increasing the dose of AA from 1000 mg to 2000 mg daily at the time of acquired resistance to standard-dose therapy.
- Lower baseline DHEA levels and lower abiraterone pharmacokinetic levels are associated with resistance to treatment, and further studies are warranted to determine the utility of these biomarkers in selecting patients for therapy.

Acknowledgments

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Disclosure

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Supplemental Data

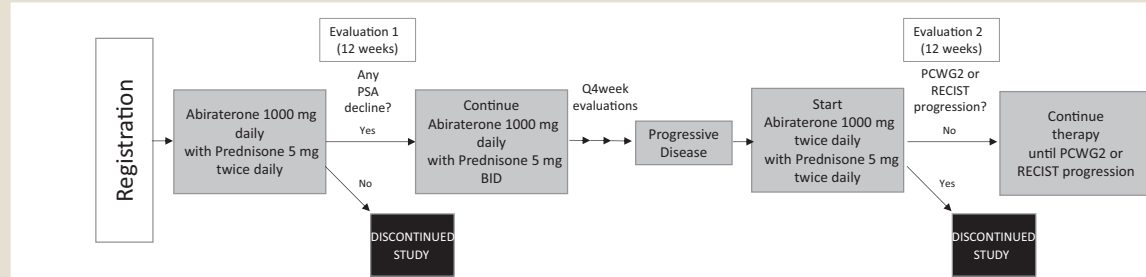
Supplemental figures accompanying this article can be found in the online version at <http://dx.doi.org/10.1016/j.clgc.2017.05.026>.

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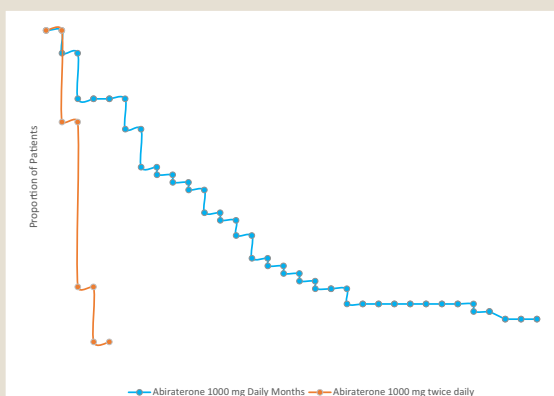
High-Dose AA in Men With CRPC

Supplemental Figure 1 Study Schema



Abbreviations: PCWG2 = Prostate Cancer Working Group 2; Q4week = every 4 weeks; RECIST = response evaluation criteria in solid tumors.

Supplemental Figure 2 Time to Prostate-Specific Antigen (PSA) Progression for Abiraterone Acetate With Prednisone-Naïve Subjects Receiving Standard-Dose (Blue) and for Abiraterone Acetate With Prednisone-Resistant Subjects Receiving Increased Dose (Red)



An improved CTC isolation scheme for pairing with downstream genomics - demonstrating clinical utility in prostate, lung and pancreatic cancer

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ABSTRACT

Metastatic biopsies are often not clinically warranted nor feasible in oncology. Isolation and subsequent genomic profiling of circulating tumor cells (CTCs) from patients with advanced cancer is a fairly non-invasive alternative for understanding tumor progression and treatment resistance and monitoring. However, most of the current, classical CTC isolation techniques cannot capture mesenchymal or epithelial-mesenchymal transition (EMT) expressing CTCs and most yield poor-purity CTCs with high leukocyte contamination. These low purities can severely limit downstream molecular and genomic analysis, and profiling epithelial cells may miss other aggressive cell populations. We have previously demonstrated the use of a cell-adhesion matrix (CAM) platform to capture invasive CTCs (iCTCs) in both a cell surface marker- and size-independent manner. The CAM method captures iCTCs based on their ability to invade a fluorescently-labeled matrix, thus allowing for capture of CTCs expressing different antigens. To improve the purity of the isolated iCTCs, in this study we used fluorescence assisted cell sorting (FACS) in combination with the CAM assay (CAM+FACS) to facilitate easy coupling with conventional downstream genomic assays. Cell line and patient samples were included from three different cancer types; metastatic castration resistant prostate cancer (mCRPC), metastatic non-small cell lung cancer (NSCLC) and metastatic pancreatic ductal adenocarcinoma cancer (mPDAC). Our results showed a significant increase of median purity from 0.04% (CAM assay) to 86% (CAM+FACS) for spiked-in prostate cancer cells (PC3) ($p < 0.0001$), 0.020% to 74% ($p = 0.0004$) for spiked-in NSCLC cell lines (HCC 827 parental and ER3) and from 0.65% to 69.3% ($p = 0.08$) for spiked-in pancreatic cancer cell lines (BxPC3 and MiaPaCa2). Similarly, purity with CAM+FACS vs CAM assay alone enriched iCTCs from patient samples from 0.39% to 90% ($p < 0.0001$) for mCRPC patients, 0.01% to 75.5% ($p = 0.0038$) for NSCLC patient samples and from 0.05% to 69.2% ($p = 0.01$) for mPDAC patients. Whole genome analysis of the high purity iCTCs recovered from spiked-in prostate cancer cell lines and a patient sample demonstrated tumor specific copy number changes associated with mCRPC. In addition, multiple genomic aberrations observed in the iCTCs isolated from a mCRPC patient sample were similar to those observed in the matched biopsy. Using allele-specific PCR and an allele-specific antibody, a clinically relevant somatic epidermal growth factor receptor (EGFR) mutation specific to NSCLC was observed in the high purity iCTCs recovered from both the EGFR+ NSCLC cell lines and patient samples. Next-generation sequencing (NGS) of high purity iCTCs isolated from a spiked-in pancreatic cancer cell line showed the expected mPDAC specific mutations. NGS of iCTCs from one mPDAC patient sample showed tumor-specific mutations such as KRAS which are characteristic of mPDAC which were not reflected in the mutations observed in the matched germline DNA. In summary, this two-step enrichment process combining CAM assay with FACS addresses key challenges of CTC isolation by using antibody-independent iCTC isolation paired with high CTC purification enabling multiple downstream genomic characterizations across different tumor types.

INTRODUCTION

Circulating tumor cells (CTCs) are rare cells found in the peripheral blood, which detach from solid tumors and intravasate into the vasculature. CTCs have multiple potential clinical applications, including allowing clinicians to better establish prognosis as well as to predict and monitor therapeutic response [1-3]. Compared to metastatic biopsies, which are often painful, labor intensive, expensive and often are absent of tumor material,

CTCs offer an opportunity to study advanced cancer in a less invasive manner with a simple blood draw which allows for serial monitoring of a cancer patient during treatment.

The only FDA-cleared CTC enrichment tool, CellSearch (Veridex), requires cells to express the epithelial cell adhesion molecule (EpCAM). While CellSearch is useful for predicting prognosis in a number of solid malignancies, it only detects cells with an epithelial phenotype and misses cells in circulation that have undergone the epithelial to mesenchymal transition (EMT), and thus no longer express EpCAM [4]. In addition, cells counted using the Veridex system are not clearly suitable for genomic analysis and suffer from low purities (< 0.3%)[5]. To address this a novel methodology that captures invasive CTCs (iCTCs) in both a cell surface marker- and size-independent manner based on the biological proclivity of metastatic tumor cells to invade a collagenous cell-adhesion matrix (CAM) has been developed. Recent work from our group has demonstrated that multiple CTC subtypes can be identified using this CAM-based CTC enrichment technology [6], including CTCs expressing markers of EMT as well as markers of stemness. In addition, we have shown that the CAM assay platform allows for isolation of CTC DNA suitable for genomic analyses [7]. However, regardless of the means of CTC isolation (i.e. phenotype, cell surface markers, size, density, and deformability) the purity of isolated CTCs can be quite variable [8]. Indeed, one of the major obstacles in performing downstream molecular and genomic analyses of CTCs is contamination with cells derived from the hematopoietic lineage (HL). Leukocytes, the largest contaminant, often decrease the purity of the isolated CTCs to as low as 0.02% [9]. Given that the CTC field is rapidly moving beyond enumeration, recovering CTCs with high purity is critical for performing reliable downstream genomic analysis.

In the current study, we sought to maximize iCTC purity by coupling the CAM iCTC isolation assay with fluorescence assisted cell sorting (FACS) to improve the quality of isolated iCTCs for downstream genomic analysis. In addition to cancer cell lines, we investigated whether this 2-step enrichment process (CAM+FACS) could be used with clinical samples (metastatic castration-resistant prostate cancer (mCRPC), non-small cell lung cancer (NSCLC) and metastatic pancreatic cancer (mPDAC) to provide clinically relevant, tumor specific mutation information. The goal of this two-step CTC enrichment approach is to offer the ability to isolate and better genomically characterize high purity iCTCs, to expand our understanding of metastasis and drug resistance.

MATERIALS AND METHODS

Cell lines

The human prostate cancer cell line PC3 was purchased from the American Type Culture Collection (ATCC). The NSCLC cell lines, HCC 827 and HCC 827 ER3 are derived from lung adenocarcinoma and have an acquired mutation in the epidermal growth factor receptor (EGFR) tyrosine kinase domain (E746 - A750 deletion) [10]. HCC 827 ER3, in comparison to the parental cell line, is an erlotinib (EGFR tyrosine kinase inhibitor) resistant cell line, [11] but they both contain the heterozygous EGFR exon 19 deletion. The NSCLC cell lines were generously provided by Dr. Trever Bivona at University of California, San Francisco (UCSF). The mPDAC cell lines (BxPC3 and MiaPaCa2) were generously provided by Dr. Eric Collisson at UCSF. The BxPC3 cell line contains the wild-type RAS whereas the MiaPaCa2 cell line contains the KRAS mutant. All the cell lines were cultured in either RPMI-1640 or DMEM medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37° C in a 5% CO₂ incubator.

Patient samples

This research study was conducted at UCSF with approval from the institutional review board. During the patient's routine clinic visit, 20 ml of peripheral blood was collected in Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ; green top, lithium heparin as anticoagulant) and processed immediately upon arrival in the

laboratory. Donor blood for spike-in experiments was taken with consent and IRB approval from healthy individuals without known malignancy.

Patients with mCRPC (patients #1, 2, 3) were partaking in either a StandUp to Cancer/Prostate Cancer Foundation West Coast “Dream Team” (WCDT) trial (NCT02432001, PI: Eric Small, UCSF) or a UCSF-led dose-escalation study of abiraterone acetate (NCT0163740, PI: Terence Friedlander). Cells from the biopsy sample (performed at the same time as that of the blood draw according to the SU2C dream team protocol) from the mCRPC patient (patient#4), whose blood was also collected for CTC enrichment, were laser captured and processed as previously described [12]. Patients with lung adenocarcinoma possessed the EGFR del 19 mutation. EGFR del 19 mutation for NSCLC patients were detected with the FDA approved cobas® EGFR Mutation Test (Roche Molecular Diagnostics). This test qualitatively detects the exon 19 deletion in DNA derived from formalin-fixed paraffin-embedded (FFPE) patient tissue. Approximate tumor percentage within the profiled lung tumor section, as determined by a board certified pathologist, was greater than 40%. The first lung cancer patient (patient#5) was a 56 year old female with EGFR exon 19 deletion positive metastatic lung adenocarcinoma whose blood was collected for iCTCs prior to afatinib treatment. The second lung cancer patient (patient#6) was a 72 year old female with EGFR exon 19 deletion mutant metastatic lung adenocarcinoma who was on erlotinib treatment for a year before her blood was collected for iCTC enrichment. The mPDAC patient (patient#7) whose iCTCs were analyzed using NGS was a 67 year old male with metastatic pancreatic adenocarcinoma whose blood was collected for iCTCs while on treatment with FOLFIRINOX (5-fluorouracil, irinotecan, oxaliplatin) at the time of blood draw.

Two-step CTC enrichment (CAM+FACS) for increased purity

Step 1: CAM assay

The following changes were made to the published CAM assay [6], spike-in experiments using cell lines (PC3, HCC 827, HCC 827 ER3, BxPC3 and MiaPaCa 2) were carried out, 4000-6000 cells were spiked into 3 ml of healthy donor blood. For all samples, blood (20 ml for patient sample) was incubated with red blood cell (RBC) lysis buffer (1:25 ratio) in a 50 ml conical tube for 5 minutes on a rotator. Nuclear cells were pelleted by centrifugation at 1000 rpm for 5 minutes. The lysis step was repeated. The cells were re-suspended and cultured with Complete Cell Culture (CCC) media (1:1 mixture of Dulbecco's modified Eagle's medium and RPMI 1640 medium supplemented with 10% calf serum, 10% Nu-serum, 2 mM L-glutamine, 1 unit/mL penicillin, and 10 µg/mL streptomycin) for 24 hours in a Vita-Assay™ AR6W (Alexa 488-CAM coated) plate (Vitatex) at 37°C. After overnight incubation, non-adherent cells were removed and fresh CCC media was added to the wells. iCTCs were released from the plates using cell releasing CAM enzyme (Vitatex).

Step 2: Flow cytometry sorting

CAM-enriched cells from Step 1 were washed with phosphate buffered saline (PBS) plus 0.2% bovine serum albumin (BSA) buffer and stained with APC anti-CD45/CD14 (HL) antibodies and DAPI. Cells were prepared for flow cytometry by filtering through a cell-strainer cap (Becton Dickinson) to remove excess non-cellular particles. The samples were analyzed by a FACS ARIA III (BD Biosciences) equipped with a 407nm, 488nm, 561nm, and 633nm lasers. iCTCs were identified as CAM^{high}/CD45/14^{low} cells. DAPI was used to exclude dead cells. Ten cell aliquots were sorted into 0.2 ml PCR tubes placed in a 96 well plate holder. For cell lines, where DNA was desired without amplification, the iCTCs were directly collected into a 1.5 ml Eppendorf tube containing the DNA lysis buffer. Prior work by our group has demonstrated epithelial prostate specific antibody staining for positive identification of prostate cancer iCTCs [6]. For the NSCLC patient samples, Pan CK and Napsin were used to identify epithelial and lung iCTCs. CK19 and CK20 were used as markers to identify epithelial CTCs in the mPDAC patient sample. Cell counts and backgating was done with the FlowJo software (Tree Star, Inc, Ashland OR).

iCTC whole genome amplification (WGA)

WGA was performed on samples prior to copy number analysis and NGS. To prevent loss of genomic material, whole cell lysis of isolated iCTCs was performed in the same PCR tube that CAM-avid cells were sorted into during FACS. Genomic DNA in the whole cell lysate served as a template for WGA. For aCGH, the used the Picoplex WGA™ Kit (Rubicon Genomics) was used, based on prior experience with WGA and aCGH [13]. For NGS, Seqplex WGA™ Kit (Rubicon Genomics) was used for the mPDAC patient sample and RepliG Ultrafast mini kit (Qiagen) was used for the spiked-in corresponding pancreatic cell line as RepliG did not amplify the patient derived iCTCs as compared to cell line samples. All these kits were used based on the manufacturer's protocol. Amplified DNA concentrations were determined with a NanoDrop™ UV-Vis spectrophotometer. All DNAs were of sufficient quality and quantity for genomic analysis as evident by UV-Vis spectrophotometry (data not shown).

DNA extraction

DNA from CAM-avid iCTCs, cell line controls and germline was extracted with a QIAamp DNA mini kit (Qiagen). The manufacturer's protocol for DNA extraction was followed. The final product was purified using a Qiagen PCR Purification Kit. All DNAs were of sufficient quality and quantity for genomic analysis by UV-Vis spectrophotometry (data not shown).

Array Comparative Genomic Hybridization (aCGH)

aCGH was carried out using a genome-wide oligonucleotide microarray platform (SurePrint G3 Human CGH 8x60K microarray kit, Agilent Technologies, Santa Clara, CA, USA), following the manufacturer instructions. Commercially available male DNA (Promega, Madison, WI, USA) was used as a reference. Samples were labeled with the SureTag DNA labeling system (Agilent Technologies) according to the manufacturer's instructions. In brief, 500 ng of test DNA and reference DNA were differentially labeled with dCTP-Cy5 or dCTP-Cy3. Arrays were hybridized for 17 hours at 65°C and 20 rpm. Slides were scanned using an Agilent microarray scanner (model GC2505C), and images were processed using Feature Extraction 3.0.1.1 and Cytogenomics 3.0.1.1 software's (Agilent Technologies).

Allele specific EGFR exon 19 deletion assay

The presence of the EGFR exon 19 E746-A750 deletion was detected in NSCLC samples by bidirectional PCR amplification (Bi-PASA), as previously described [14]. Briefly, two allele-specific primers complementary to the mutant variant sequence and two outer primers that flanked the variation site were used. The outer primers, designated 19P and 19Q (5'-GTAACATCCACCCAGATCACTG-3' and 5'-GTGTCAAGAACTAGTGCTGGG-3'), were designed to detect a 444bp amplicon that served as an internal standard. The inner allele specific primers, designated 19A and 19B (5'-CCCGTCGCTATCAAGGAATTAA-3' and 5'-GTTGGCTTTTCGGAGATGTTTTGATAG-3'), were designed to detect homozygous mutant DNA (19AB) or the heterozygous genotype, which is characterized by 325bp (19AQ) and 135bp (19PB) fragments respectively. The PCR reaction was performed with 200ng genomic DNA; 200µmol/l dNTP, 0.25µmol/l of primers, 1.5mmol/l MgCl₂, and 0.5 U *Taq* polymerase. Cycling conditions were as follows: 5min of denaturing at 94 °C and 30 cycles of 94 °C for 30s, annealing at 58°C for 45s and elongation at 72°C for 1min. PCR products were visualized by gel electrophoresis.

Next-generation sequencing (NGS) analysis

MiaPaCa 2 stock cell line DNA, MiaPaCa 2 spiked in DNA enriched through CAM assay only, amplified DNA from MiaPaCa 2 spiked in enriched through CAM+FACS along with the amplified DNA from isolated high

purity iCTCs from mPDAC patient sample (patient #7) and the matched germline DNA went through target enrichment, library preparation, and sequencing using a targeted cancer panel (AmpliSeq CHPv2, Ion PGM, Thermo Fisher). This panel is designed to amplify 207 amplicons covering approximately 2,800 COSMIC mutations from 50 oncogenes and tumor suppressor genes. NGS was done at the UCSF Genome Core. Final variants were filtered using Ion Reporter software and a specialized set of filters as published in our previous paper [15] which included strand bias, homopolymer length, and exclusion of common germline mutations with the exception of allele ratio and allele read counts which were both increased since the purity of this CTC isolation was considerably higher.

Statistical Analysis

Student's t-test was performed to compare the purity between iCTCs isolated after CAM assay alone and CAM+FACS. Results with two-tailed P-values less than 0.05 were considered statistically significant.

For comparison between different aCGH profiles, copy number (CN) log₂ intensity values were mapped to the human genome sequence hg19 freeze. Probes with missing annotation were removed and then duplicate probes were averaged. To reduce the noisiness in the data, it was then averaged using 10 probes in non-overlapping windows within each chromosome. The CN values were then segmented using circular binary segmentation to translate noisy intensity measurements into regions of equal CN [16]. The sequential-order difference median absolute deviation (MAD) estimates, scaled by the factor 1.4826, as implemented in the matrixStats package in R [17] of the CN values was used to estimate the sample-specific experimental variation, after excluding the probes from X and Y chromosomes. Agreement between a pair of samples was measured by Cohen's kappa statistic based on gain/loss/normal status of the probes. Kappa score (poor agreement = less than 0.20, fair agreement = 0.20 to 0.40, moderate agreement = 0.40 to 0.60, good agreement = 0.60 to 0.80, very good agreement = 0.80 to 1.00). The Y chromosome was excluded when calculating these statistics.

All analyses were done using R [18] and Bioconductor software [19] in the UCSF Computational Biology Core.

RESULTS

CTC purity

CTCs from spiked-in cell lines and patient samples were recovered using the CAM assay, and then subjected to FACS purification. Cell line spike-in experiments were repeated in duplicate or triplicate. To calculate purity of the cells isolated by CAM+FACS, the recovered cells were subjected to repeat FACS analysis. Coupling of the CAM assay with FACS yielded higher purity iCTCs for all cell line and patient samples tested. With CAM enrichment alone, the median purity (defined as iCTC/(iCTC+HL) x 100%) of mCRPC cell line recovered after spike-in was 0.04% (range 0.02-0.05) NSCLC cell lines 0.020% (range 0.017-0.023), PanCan cell lines 0.645% (range 0.09-1.2). For patient samples, the median purity of iCTCs enriched by CAM assay was 0.39% (range 0.35-1.43) for mCRPC, 0.0095% (range 0.009-0.01) for NSCLC and 0.05% for mPDAC (range 0.03-0.07). Median purity of iCTCs after CAM+FACS significantly increased to 87.1% (p < 0.0001) for spiked-in mCRPC cells, 75% (p = 0.0004) for spiked in NSCLC cells, and 69.25% (p = 0.08) for spiked in PanCan cells. Similarly, isolation of iCTCs using CAM+FACS increased the iCTC median purity for patient samples as well (93.3% (p < 0.0001) for mCRPC, 71.1% (p = 0.0038) for NSCLC, and 69.25% (p = 0.01) for mPDAC (Table 1).

CTC subpopulation analysis for NSCLC patients and mPDAC patients

The iCTCs enriched from the NSCLC patient samples using CAM+FACS were also positive for cytokeratin (CK), 62% of the enriched CTCs/7.5 ml blood tested positive for CK for patient#5 and for patient#6, 42% of the CTCs enriched were positive for CK. In addition to CK+ iCTCs, Napsin+ iCTCs were also detected in this patient blood sample.

The iCTCs enriched from the mPDAC patient sample (patient#7) on a separate blood draw (taken during patient's clinic visit on a different day) also tested positive for cytokeratin (CK 19 and 20), 5 of the 5 enriched CTCs/7.5 ml blood tested positive for CK.

GENOMIC ANALYSIS OF HIGH PURITY iCTCs

aCGH-based copy number analysis of high-purity iCTCs

PC3 mCRPC cells were used as a control to evaluate the coupled CTC enrichment assay (CAM+FACS) for use with aCGH because the copy number profile of this cell line is well characterized. The aCGH profile of the PC3 cell line was fully consistent with published results for this cell line [20]. Kappa scores were computed and used to compare the different sample groups (Table 2).

Prior studies have raised doubts that whole genome amplification methods may generate nonspecific amplification artifacts which cannot be used in many down-stream applications[13]. To address this we evaluated the performance of Picoplex kit using PC3 cells. The amplification method maintained good correlation for the copy number data obtained from unamplified PC3 stock DNA versus amplified PC3 stock DNA (Case 1, Table 2). Compared to amplified stock DNA, the correlation was fair for amplified cells isolated via CAM+FACS (Case 2, Table 2). A good correlation (kappa score 0.6) was obtained when comparing amplified 10 cells to those isolated with CAM+FACS (Case 3, Table 2).

This demonstrated that post CAM+FACS, direct amplification of a small number of cells (iCTCs) was better when compared to amplified 10 cells rather than amplified stock DNA. Figure 1A demonstrates representative aCGH plots of the PC3 samples from Table 2. It should be noted that aCGH of DNA extracted from cells directly after CAM isolation alone did not pass quality control and thus were not included in Table 2.

To demonstrate the clinical utility of the high purity iCTCs enriched using the coupled CTC enrichment, iCTCs from a mCRPC patient sample (patient#4) were analyzed with aCGH. Tumor cells from a paired metastatic biopsy from the same patient and analyzed on the same platform were included for comparison purposes. The biopsy was performed at the same time as the CTC blood draw. Both iCTCs and cells laser-captured from the paired metastatic biopsy were subjected to whole genome amplification and aCGH to determine whether tumor specific copy number changes observed in the biopsy sample were reflected in the iCTCs. The aCGH analysis of the iCTCs revealed genomic aberrations characteristics of prostate cancer, including losses in chromosomes 5q, 6q, 8p, 10q, 13q, 16q, 17p, and 18q, and gains in 7p/q, 8q, 9p, and Xq. (Figure 1B). The observed changes characteristic of mCRPC, confirm the malignant nature of the iCTCs. Comparison of the respective genomic profiles (iCTCs and the biopsy) revealed shared copy number aberrations indicative of common lineage, including losses in 8p, 13q and phosphatase and tensin homolog (PTEN) (Figure 1B). Interestingly, despite the fact that androgen receptor (AR) amplification is commonly present in mCRPC, neither the iCTC DNA nor the matched biopsy showed amplification at the AR locus [21, 22]. The copy-neutral AR findings from the patient biopsy tissue sample were also confirmed by fluorescence in situ hybridization (FISH) (data not shown). Copy number aberrations observed in the iCTCs but not in the primary tumor, included gain at the 8q locus (Figure 1B).

Clinically relevant gene mutations detectable in high-purity iCTCs using PCR

EGFR is commonly mutated in NSCLC and is often used as part of the clinical assessment. An allele-specific PCR assay for EGFR was evaluated on NSCLC cell lines spiked into healthy donor blood. HCC 827 NSCLC cells are known to harbor an exon 19 EGFR deletion [23], which is frequently observed in NSCLC. The mutant allele was robustly detected for the HCC 827 and HCC827 ER3 cell lines recovered with the CAM+FACS

(Figure 2, lanes d and e), compared to CAM assay alone (Figure 2, lanes b and c) which showed a fainter band. Amplified DNA from high purity iCTCs isolated with CAM + FACS from two different NSCLC patients known to harbor the EGFR exon 19 mutation was tested and the mutant allele was detected for both (Figure 2, lanes g and i). However, the matched germline DNA (Figure 2, lane h) from patient #6 showed a fainter band indicating some degree of non-specificity of the allele specific primers. So additional confirmation of the same EGFR mutation was carried by an independent assessment where CAM assay enriched iCTCs for NSCLC patient samples were FACS sorted with exon 19 EGFR E746-A750 deletion specific antibody. 30% of the iCTCs enriched were positive for EGFR E746-A750 mutation specific antibody was detected.

NGS based mutation detection with high-purity iCTCs

Pancreatic cancer has a high rate of single base changes, include those in KRAS. Most of these genomic alterations are represented in cancer-focused sequencing panels. DNA was isolated from the mPDAC cell line MiaPaCa, and iCTCs were isolated from a spike in experiment using the CAM assay alone and CAM+FACS, and results were analyzed using NGS (AmpliSeq CHPv2, Ion PGM, Thermo Fisher). The cell line DNA showed the expected KRAS, TP53 and NOTCH1 mutations [24]. These three mutations were also observed in the MiaPaCa spiked in CAM+FACS sample, but not in the sample processed with the CAM assay alone. The allele ratios for all the mutations observed in the cell line DNA and MiaPaCa 2 spiked in CAM+FACS samples were found to be ≥ 0.9 (Table 3).

In the mPDAC iCTC patient sample (patient#7), processed by CAM+FACS, 14 cancer gene mutations were observed (Table 4), however no cancer tissue was available for comparison. This included KRAS, which is commonly mutated in PanCan. Thirteen out of the 14 iCTC mutations were not shared with the germline sample.

DISCUSSION

In this study we sought to address a technological challenge faced by many CTC enrichment platforms: how to efficiently isolate high purity CTCs suitable for downstream genomic analysis. We have previously shown that the marker-independent capture of iCTCs using the CAM assay allows for the isolation of multiple CTC subpopulations [6]. Prior studies using this assay have demonstrated isolation of CAM-avid cells of epithelial origin ($>80\%$) in breast [25, 26][24, 25][23, 24][22, 23][20, 21] and ovarian cancer [27]. Here, we sought to improve the iCTC purity for genomic analysis by combining the CAM assay with FACS sorting. An added benefit of using FACS is that it is semi-automated and, compared to microscopy, is less subject to observer bias. CTCs isolated from cell lines and blood of patients with three different types of cancers, mCRPC, NSCLC and mPDAC, were included for illustration purposes. This is the first demonstration of using the CTC platform for enriching iCTCs from NSCLC and mPDAC.

iCTC purity measurements were carried out to evaluate the benefit of adding FACS to the CAM assay. Cell lines from all three tumor types (prostate, lung and pancreatic) spiked into healthy donor blood and recovered on the CAM assay showed that the CAM+FACS allows for iCTC purities as high as 92% (range 86-92%) for prostate cancer cells, 77% for lung cancer cells (range (74-77%)) and 90.6% for pancreatic cancer cells (range (47.9-90.6%)) (Table 1). In all cases, this was a significant improvement in purity from the sample recovered with the CAM assay alone. It should be noted that the purity of the pancreatic cancer cell lines varied depending on which cancer cell line was tested. To the best of our knowledge, the purity of CTCs obtained by our CAM+FACS is one of the highest reported in the literature, especially for prostate cancer. For comparison other commonly used patient-derived CTC isolation techniques have purity percentages ranging from as low as 0.02% [28, 29] to 60% [30]. The difference in the purity between different patient samples and cell line spiked-in samples could be attributed to the inter-individual differences in the numbers of peripheral white blood cells.

It is noteworthy that this is the first demonstration that NSCLC and mPDAC CTCs (both cell lines and patient samples) are suitable for isolation on the CAM platform and were of high-purity. Interestingly, both NSCLC cell lines i.e. parental/EGFR-inhibitor naïve, HCC 827, and EGFR-inhibitor resistant cells, HCC 827 ER3, were recoverable using the CAM assay. In line with the CAM-based invasion assay, the EMT+ NSCLC cells (HCC 827 ER3) were more CAM-avid than the EMT- parental NSCLC cell line (HCC 827) (Table 1). Most commonly used CTC enrichment techniques rely on antibody-mediated selection of classic epithelial markers such as EpCAM and CK expressing CTC cells which may be unable to identify CTCs that have undergone EMT [31-33]. Since the presence of EMT and stem-like cells are linked to more aggressive clinical behavior [34-37] and may alter drug sensitivity [38], it's important to have a CTC enrichment tool that allows for their isolation. CAM-avid lung iCTCs also stained positive for Pan CK and Napsin A (data not shown). Napsin A is a cytoplasmic stain that is relatively specific for adenocarcinoma of the lung and reportedly stains 80% of cases [39, 40]. In addition, iCTCs isolated with CAM+FACS for both of the NSCLC patient samples were positive for the EGFR mutation regardless of disease stage and treatment. Thirty percent of iCTCs enriched via CAM+FACS from a NSCLC patient sample also stained via FACS for exon 19 EGFR E746-A750 deletion specific antibody which has been shown to have high sensitivity and specificity and is being considered suitable for screening of this particular mutation [41, 42]. Similarly, CAM-avid pancreatic iCTCs also tested positive for CK19 and CK20 which have been reported to be highly expressed in pancreas cancer [43] indicating that iCTCs isolated from mPDAC patient samples using the CAM assay likely represents true pancreatic cancer cells. In addition, a KRAS mutation which is present in >85% of pancreatic cancers was also found to be one of the mutations identified in the iCTC DNA of the patient sample [44]. Altogether this demonstrates that the iCTCs isolated using the CAM assay indeed represents NSCLC and mPDAC tumor cells. We have previously shown that the iCTCs isolated from mCRPC patients are of prostate origin as these CAM derived iCTCs stained positive for Prostate-specific antigen (PSA) / Prostate-specific membrane antigen (PSMA) [6].

Having successfully isolated iCTCs from three different tumor types using CAM+FACS, we assessed whether these iCTCs were suitable for different downstream genomic assays, i.e. aCGH, PCR and NGS. To demonstrate the clinical application, iCTCs isolated from mCRPC patient samples were analyzed with aCGH, iCTCs from NSCLC patient samples were analyzed using allele specific PCR and NGS was used to analyze iCTCs isolated from an mPDAC patient sample. aCGH was selected for the genomic assay for the prostate cancer samples as this is a clinically relevant downstream genomic assay for mCRPC since copy number aberrations are common mutational events in prostate cancer [13, 45, 46]. PCR was chosen for the genomic assay for the lung cancer samples because of the well-characterized, translationally relevant mutation in the EGFR oncogene. FACS also allows for antibody-based mutation detection. NGS was selected for the genomic assay for the pancreatic cancer sample because somatic mutations are very common in pancreatic cancer tumors. iCTCs isolated from the mCRPC patient revealed a wide range of copy number aberrations including those that have been previously reported to be characteristic of prostate cancer. For example, gain of 8q is one of the most common genetic aberrations in mCRPC [22]. The aCGH profiles of the isolated iCTCs did not appear dampened by normal DNA from HL cells as demonstrated by the PC3 cell line experiments which showed a good correlation when amplified PC3 cells were compared to those isolated with CAM+FACS, further demonstrating the high purity of the iCTCs isolated using our combined approach. Since CTCs are rare in most patients, the number of cells required to obtain sufficient quantity of genomic DNA for DNA extraction is often not sufficient and combining large pools of CTCs may not reflect the heterogeneity of CTC populations. Therefore, we explored using minimal cell input. We collected multi-cell aliquots (10 cells), which were then subjected to whole genome amplification. Although whole genome amplification of DNA can introduce artifacts, it was observed that overall genomic aberrations were retained during DNA amplification of CAM+FACS enriched cells (Table 2, Figure 1A) consistent with our prior work in DNA amplification [13]. Comparisons of iCTCs with matched archival primary tumors also confirmed a degree of shared lineage such as loss of PTEN (Figure 1B) The presence of PTEN mutations has been associated with poor prognosis and higher rate of metastasis in prostate cancer [47-49]. Despite many similarities, there were some differences observed between matched tissue and iCTCs aCGH profiles. These differences could be attributed to tumor heterogeneity, in that the isolated iCTCs may have been derived from a different primary or metastatic site than the metastatic site that was biopsied, or

amplification artifact. Multiple, recent studies [50] have documented the presence of tumor heterogeneity in metastatic malignancies and may therefore explain some of the differences observed in our study as well. Further studies in larger numbers of patients need to be conducted in order to explore this further. Overall CAM+FACs provides iCTCs suitable for aCGH.

Somatic mutations in the EGFR have been detected in approximately 10% of NSCLC patients and are associated with sensitivity to erlotinib treatment. Of these somatic EGFR mutations, 90% are nucleotide deletions around codons 746-750 in exon 19 [51, 52]. This activating mutation in the tyrosine kinase domain plays an important role in lung oncogenesis and tumor progression and is related to the clinical efficacy of EGFR tyrosine kinase inhibitors such as erlotinib. As a result, analysis of these mutations has become an important tool for targeted therapy in lung cancer [53]. HCC 827 and ER3 NSCLC cell lines are known to harbor the exon 19 EGFR deletion. In this study it was shown that compared to CAM iCTC enrichment alone, DNA isolated from iCTCs obtained via CAM+FACS were more robustly positive for the EGFR deletion 19 (Figure 2). This was true for both of the lung cancer cell lines. These results indicate that detecting mutations, in this particular case the EGFR mutation, in tumor samples contaminated with HL cells is highly possible with high purity iCTCs isolated with the combined CAM+FACS approach. The mutational status of EGFR is a strong predictor of sensitivity to agents such as erlotinib [54-56], and detection of such mutations on CTCs could provide patients and physicians with important information for therapy selection and treatment monitoring via a liquid biopsy.

CTCs have been detected in patients with advanced pancreatic cancer albeit in very few studies, and in general the number of CTCs reported has been low [57, 58]. The low frequency of CTCs in mPDAC has been attributed to physiological reasons such as decreased vascularity and increased tissue stroma which may prevent dissemination of large numbers of cells, as well as use of CTC enrichment platforms such as Cell Search which relies on epithelial marker based isolation and thus could miss cells undergoing EMT [59-61]. iCTCs enriched via CAM+ FACS are not only independent of epithelial markers but also high in purity. Amplified DNA isolated from iCTCs obtained via CAM+FACS from the spiked in pancreatic cancer cell line showed the exact same mutations that were seen and known to exist in the cell line stock DNA when sequenced. However, DNA isolated from iCTCs enriched from CAM assay alone did not yield the same mutations as that of the cell line stock DNA possibly due to lower purity (Table 3). We were able to detect somatic mutations in the DNA from the iCTCs from a mPDAC patient sample isolated via CAM+ FACS that were not reflected in the matched germline DNA. Fourteen mutations were detected via NGS in the mPDAC patient sample including KRAS and 13 out of the 14 iCTC mutations were not shared with the matched germline sample (Table 4) suggesting that these mutations were limited to the cancer. While cancer tissue from this patient was not available for comparison, the work suggests that, with further validation, CAM+FACS enrichment of iCTCs can yields clinically relevant CTCs in different tumor types.

In summary, we show a novel approach to isolating high-purity iCTCs and demonstrate its utility with samples from patients with prostate, lung and pancreatic cancers. These purified iCTCs are suitable for various downstream genomic analyses, including PCR, aCGH and NGS. Compared to tissue biopsy this liquid biopsy approach offers a way to better isolate and characterize cancer cells to afford a better understanding of disease progression.

FIGURES: Table 1

Samples	iCTC counts (CAM assay)	iCTC counts (CAM+FACS)	WBC counts (CAM assay)	WBC (CAM+FACS)	% Purity (CAM assay)	% Purity (CAM+FACS)
PC3 spike in	1658	650	3489083	260	0.04%	86.4%
PC3 spike in	1518	173	3827958	225	0.04%	87.1%
PC3 spike in	52	11	213856	5	0.02%	92.0%
HCC 827 ER3 spike in	337	190	1430323	103	0.02%	76.5%
HCC 827 ER3 spike in	270	163	1558830	97	0.01%	73.6%
BxPC3 spike in	4835	2280	401735	502	1.20%	90.6%
MiaPaCa 2 spike in	23	11	24585	25	0.09%	47.9%
mCRPC patient blood sample (patient#1)	463	249	116253	48	0.40%	90.5%
mCRPC patient blood sample (patient#2)	1224	381	85775	89	1.42%	93.3%
mCRPC patient blood sample (patient#3)	272	122	78075	9	0.35%	96.7%
NSCLC patient blood sample (patient#5)	249	95	2466832	81	0.01%	75.5%
NSCLC patient blood sample (patient#6)	120	17	1208368	60	0.009 %	66.7%
mPDAC patient blood sample (Patient#7)	292	150	397397	82	0.07%	78.1%

mPDAC patient blood sample (Patient#8)	131	53	480385	86	0.03%	60.4%
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Table 2

	Sample comparisons	Kappa score
Case 1	Unamplified PC3 stock DNA	0.62
	Amplified PC3 stock DNA	
Case 2	Amplified PC3 stock DNA	0.28
	Amplified PC3 10 cells (CAM+FACS)	
Case 3	Amplified PC3 10 cells	0.6
	Amplified PC3 10 cells (CAM+FACS)	

Table 3

MiaPaCa 2 cell line stock DNA			MiaPaCa 2 CAM assay			MiaPaCa 2 CAM+FACS		
Genes	Locus	Allele Ratio	Genes	Locus	Allele Ratio	Genes	Locus	Allele Ratio
KRAS	Chr12:25 398285	1	MET	Chr7:1163 39642	0.522	KRAS	Chr12:25 398285	1
TP53	Chr17:75 77539	0.99				TP53	Chr17:75 77539	0.9
NOTCH1	Chr9:139 390822	1				NOTCH1	Chr9:139 390822	0.91

Table 4

mPDAC patient sample DNA	
Genes	Locus
ERBB4	chr2:212652722
CTNNB1	chr3:41266055
PIK3CA	chr3:178927460
PIK3CA	chr3:178927494
PIK3CA	chr3:178927497
MET	chr7:116403165
MET	chr7:116403188
SMO	chr7:128851587
FGFR2	chr10:123274818
ATM	chr11:108236080
KRAS	chr12:25380323
HNF1A	chr12:121431429
SMAD4	chr18:48575144
SMAD4	chr18:48575185

Figure 1A

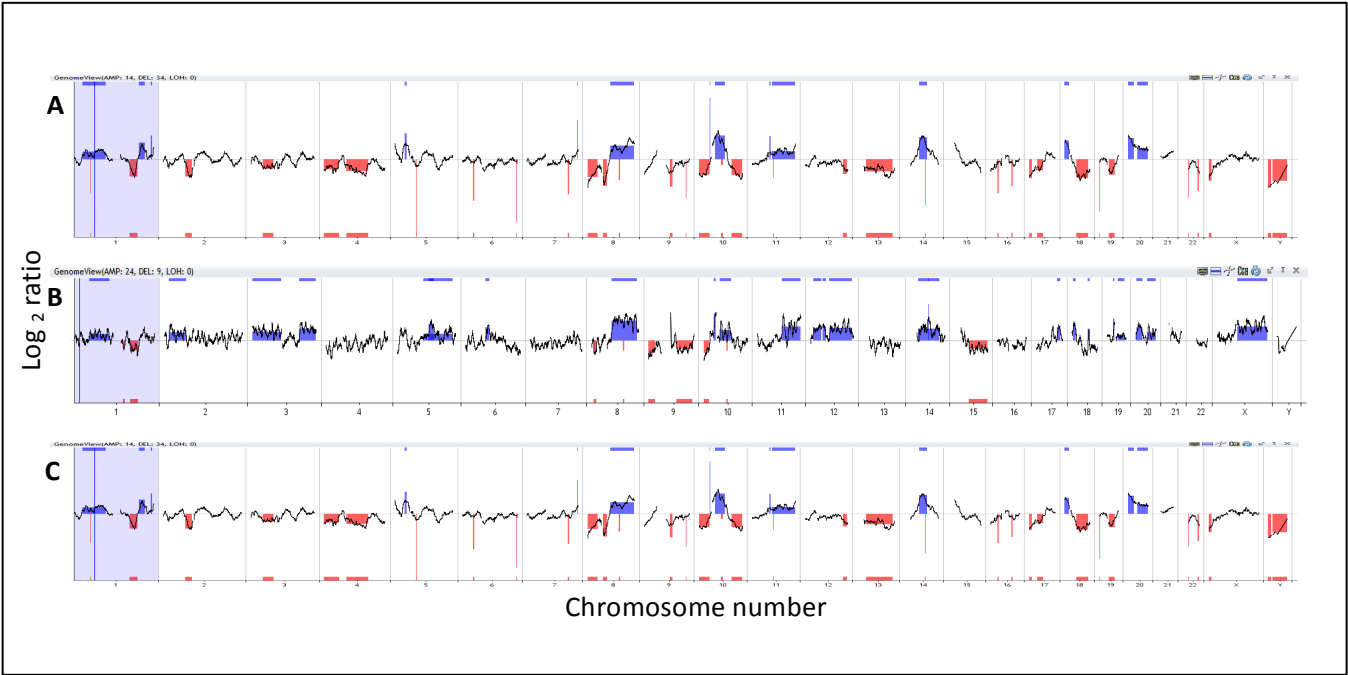


Figure 1B

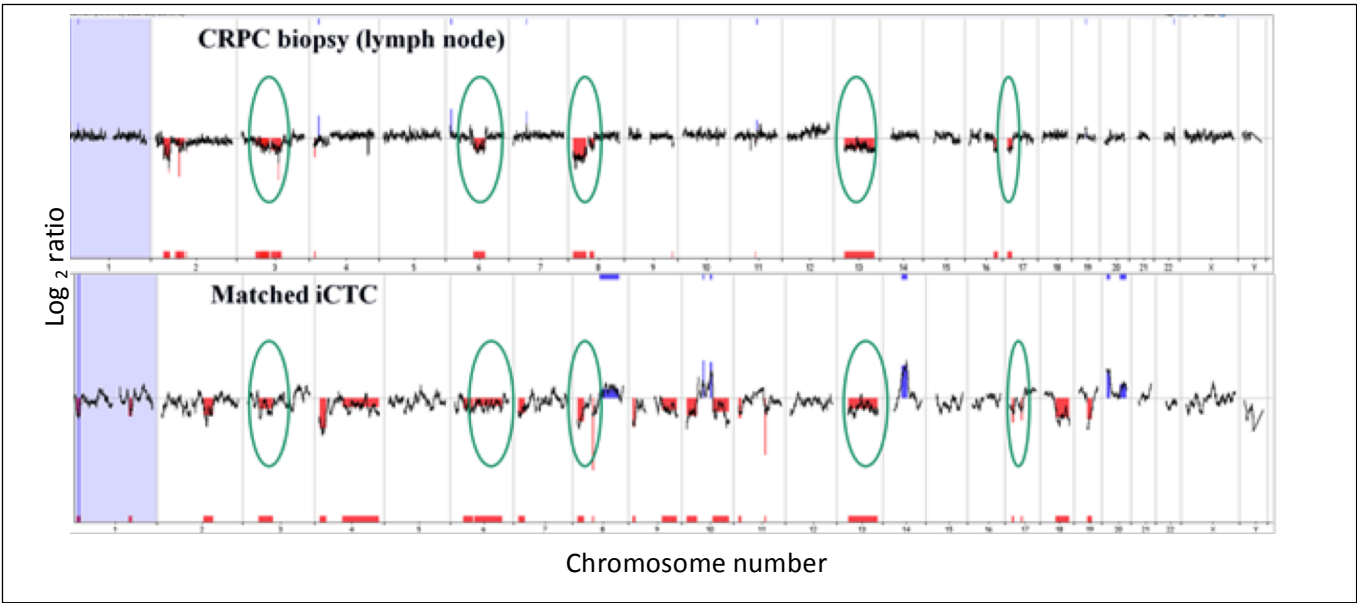


Figure 2

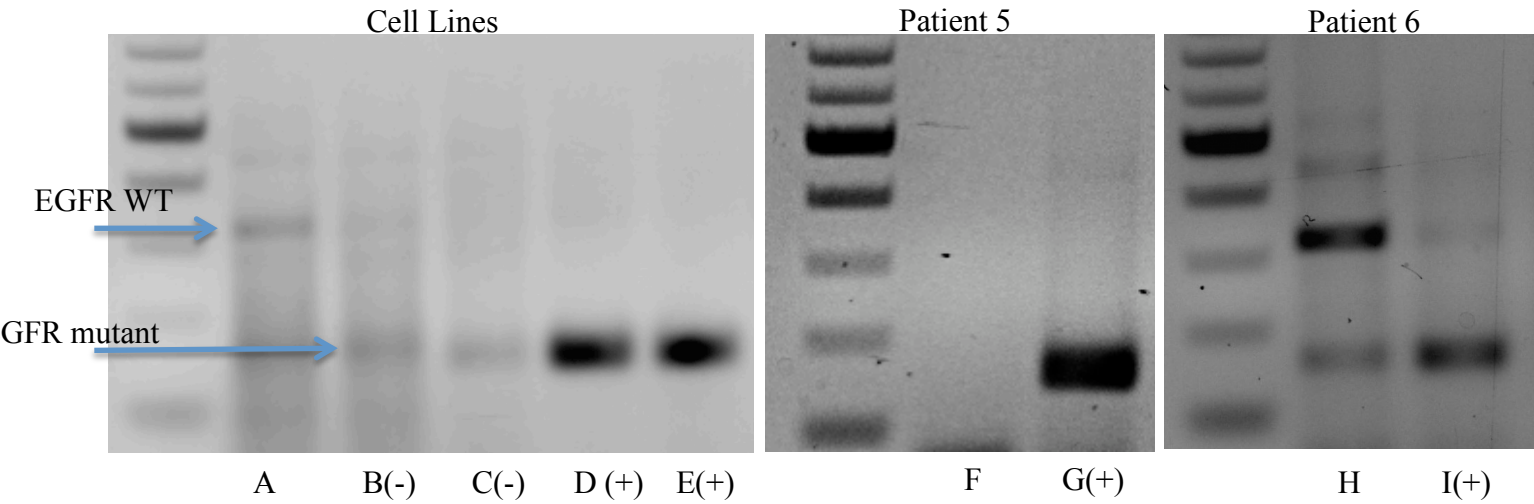


FIGURE LEGENDS

Table 1: **Coupling of CAM platform with FACS yields high-purity iCTCs.** Purity % was estimated for prostate, lung and pancreatic spiked in cell lines and patient samples using the formula, $Purity \% = (CTC\ counts / (CTC + WBC\ counts)) \times 100$.

Table 2: **CNV pattern of a pool of spiked in PC3's (10 cells) recovered from CAM+FACS after whole genome amplification was fairly similar to the CNV pattern obtained from unamplified PC3 cell line stock DNA.** Cohen's Kappa correlation analysis (refer to statistical analysis section in the methods for the agreement ranges) was carried out to using PC3 cells under different case scenarios to determine whether whole genome amplification and pooling of a small number of cells after CAM+FACS enrichment adequately represented the entire iCTC isolated population genomically.

Table 3: **iCTCs from spiked in pancreatic cell line and the matched cell line stock DNA showed similar variants.** A model pancreatic tumor cell line (MiaPaCa 2) was spiked into healthy donor blood and enriched through CAM assay only and CAM+FACS and analyzed using targeted NGS. The cell line stock DNA showed 3 known somatic variants in KRAS, TP53 and NOTCH1 genes. Each of these variants was also detected in the MiaPaCa 2 DNA isolated from iCTCs enriched via the CAM+FACS with similar allelic ratios, however the MiaPaCa 2 DNA from iCTCs enriched via CAM assay alone had only one positive call for a MET gene which was not observed in the cell line stock DNA.

Table 4: **iCTCs from pancreatic patient sample and the matched germline did not show similar variants.** iCTCs were enriched from a mPDAC patient sample (patient#7) via the CAM+FACS. DNA from them was then isolated and amplified along with the matched germline DNA and analyzed using NGS. The patient sample iCTC DNA showed 14 variants of which KRAS was one of them. Out of the 14 mutations detected in the patient iCTC DNA only one mutation (ERBB4) was also found in the germline DNA.

Figure 1A: **Representative aCGH profiles of PC3 cells. Copy number analysis of** a) amplified PC3 DNA, b) and c) amplified PC3 10 cells with and without CAM+FACS enrichment respectively. X axis represents chromosomes numbers 1-22 and x and y, whereas y axis indicates the log2 copy number ratio, log2 0= no copy aberrations; Color represents copy number status: red = copy loss, blue = copy gain.

Figure 1B: **iCTCs and the matched primary tumor showed similar copy number changes.** iCTCs (10 cell aliquot) from a mCRPC patient sample (patient#4) were collected, amplified and arrayed (top row), 50 tumor cells from the biopsy tissue were laser captured, collected, amplified and arrayed (bottom row). X axis represents chromosomes numbers 1-22 and x and y, and the y axis indicates the log2 copy number ratio; red = copy loss, blue = copy gain.

Figure 2: **iCTCs isolated from spiked in NSCLC cell lines and NSCLC patient samples contained the EGFR exon 19 with CAM+FACS enrichment. Samples enriched with CAM alone are indicated with a (-) sign and samples enriched with CAM+FACS are indicated with a (+) sign.** Somatic mutation EGFR del 19 in NSCLC derived cells and iCTCs; a) germline DNA, b) HCC 827 ER3 cells enriched from CAM assay, c) HCC 827 parental cells enriched from CAM assay, d) HCC 827 ER3 cells enriched from CAM+FACS, e) HCC 827 parental cells enriched from CAM +FACS, f) negative control (water), g) CK+ CAM+ CD45- DAPI- iCTCs isolated from an NSCLC patient (patient#5) sample using CAM+FACS, h) germline DNA from NSCLC patient sample #6, and i) CK+ CAM+ CD45- DAPI- iCTCs isolated from the NSCLC patient sample #6 using CAM+FACS. Coupling CAM+FACS robustly detects the mutation in comparison to cells evaluated after CAM assay enrichment alone.

ABBREVIATIONS

CTCs- Circulating Tumor Cells

iCTCs- invasive Circulating Tumor Cells

CAM- Cell Adhesion Matrix

FACS- Fluorescence-Activated Cell Sorting

EMT- Epithelial to Mesenchymal Transition

EpCAM- Epithelial Cell Adhesion Molecule

mCRPC- metastatic Castration Resistant Prostate Cancer

NSCLC- Non-Small Cell Lung Cancer

mPDAC- metastatic Pancreatic Cancer

EGFR- Epidermal Growth Factor Receptor

NGS- Next-Generation Sequencing

aCGH- Array Comparative Genomic Hybridization

WGA- Whole Genome Amplification

CK- Cytokeratin

PTEN- Phosphatase and Tensin Homolog

PSA- Prostate-specific antigen

PSMA- Prostate-specific membrane antigen

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